

THE ISOLATION AND CHARACTERIZATION OF THE LYMPHOCYTE
RECEPTOR FOR ANTIGEN

by

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Statement

The investigations described in this thesis
constitute my own original work and were
carried out by myself, except where otherwise
stated.

[Signature]

To my Mother and Father

Page 22
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Statement

The investigations described in this thesis constitute my own original work and were carried out by myself, except where otherwise stated.



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Chapter 1

General introduction

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This thesis is concerned with lymphocyte surface-immunoglobulins. These molecules, in particular IgM, now seem certain to be the receptors for antigen on nonthymus- and possibly also on thymus-derived lymphocytes. However, this has still to be proved unequivocally for either kind of cell.

The first part of this introduction concerns antigen-binding cells, their biological significance, and the nature of their reaction with antigen. These phenomena provide direct evidence for the existence of receptors for antigen. The second part considers the characteristics of lymphocyte surface-immunoglobulins.

The ability of animals to mount an immune response depends upon antigen-mediated reactions between lymphocytes and one of at least two types of cell, macrophages or dendritic follicular cells (Nossal and Ada, 1971). The role of macrophages and dendritic cells is nonspecific, in that neither is capable of binding antigen to its surface without the help of cytophilic antibodies (Nelson, 1969; Nossal and Ada, 1971; Feldman and Nossal, 1972). These cells are involved in antigen manipulation, such as its degradation to smaller antigenic units (macrophages only) or its localization on their surfaces. In contrast, lymphocytes are essential for *specific* recognition of antigen and are themselves responsible for tolerance or immunity (humoral and/or cell-mediated) and are capable of transferring these phenomena to lethally-irradiated recipients (Warner, Szenberg and Burnet, 1962; Gowans and Uhr, 1966; Miller and Mitchell, 1968; Weigle, Chiller and Habicht, 1972).

In the mouse and chicken, two kinds of lymphocyte have been distinguished by their different immunocompetent abilities and also, in the mouse, by their surface antigens (Roitt, Greaves, Torrigiani, Brostoff and Playfair, 1969; Raff, 1971).

Thymus-derived lymphocytes ("T" cells) can be activated by antigen to (a) initiate cell-mediated immune responses and/or (b) co-operate with B lymphocytes in the initiation of humoral immunity. In mice, T lymphocytes bear the theta (θ) alloantigen on their surface (Reif and Allen, 1964).

Nonthymus-derived lymphocytes ("B" cells) are

precursors of antibody-forming cells and are derived from the bursa (birds) or bone marrow (mice). B lymphocytes have large quantities of immunoglobulin (Ig) on their surface (*mouse*: Unanue, Grey, Rabellino, Campbell and Schmidtke, 1971; *chicken*: Rabellino and Grey, 1971; Kincade, Lawton and Cooper, 1971).

1.1 Antigen-binding cells (ABC)

It now seems certain that within any population of immunocompetent cells from normal animals, only a proportion of cells can specifically bind any one antigen (see reviews: Sulitzeanu, 1968; Ada, 1970; Roelants, 1972a). This proportion, which depends on the nature, and concentration of the antigen used, seems to have an upper limit of 5-10 % although in most instances it is less than 1 %. Antigen-binding is termed specific if (a) it occurs at 0°C in the presence of anti-metabolites (e.g. sodium azide) at concentrations which prevent phagocytic uptake of antigen, and (b) it is inhibited by the homologous or serologically-related, but not unrelated, antigens.

1.1.1 Detection of ABC

ABC have been detected by the binding of either macroscopic or soluble antigens. Macroscopic antigens are useful both for the detection of ABC and for their separation from other cells. Because of their size, however, few particles can be bound by any one ABC and the number or density of antigen receptor sites on ABC cannot be determined. Normal or antigen-coated red blood cells (RBC) have been used extensively and form rosette-like clusters with ABC, which are consequently termed "rosette-forming cells" (RFC) (Zaalberg, 1964; Laskov, 1968; Biozzi *et al.*, 1968; Greaves and Möller, 1970; Wilson, 1971; Bankhurst and Wilson, 1971; Wilson and Miller, 1971; Möller and Sjöberg, 1972; Hogg and Greaves, 1972; Ashman and Raff, 1973). Synthetic beads and fibres coated with antigen have also proved useful, especially for the removal (depletion) of ABC from cell populations (Baker, Bernstein, Pasanen and Landy, 1966; Abdou and Richter, 1969; Wigzell, 1970; Henry, Kimura and Wofsy, 1972; Rutishauser, Millette and Edelman, 1972).

The use of soluble antigens permits determination of the number of antigen molecules bound and so of the number and

density of receptors on different ABC. Radiolabeled antigens are most commonly used and ABC, after incubation *in vitro* with labeled antigen, are detected by light or electron microscope autoradiography. This method, first applied to the detection of ABC by Naor and Sulitzeanu(1967) has since been used by numerous workers using a variety of antigens and animals (see reviews: Sulitzeanu,1968; Ada,1970; Roelants,1972a). The proportion of cells identified as binding an antigen depends on factors such as the concentration and specific radioactivity of the antigen, duration of exposure of autoradiographs, number of cells counted, and the criteria used to designate a cell positive (Ada,1970). Enzymes have also been employed, their catalytic activity being used to identify cells which bind them as antigens (Modabber and Sercarz,1970; Rotman and Cox,1971).

The results obtained using either type of antigen (soluble or macroscopic) are qualitatively similar and in general show that:

- (a) Antigen binds specifically to the surface of ABC, in the presence or absence of antimetabolites and at temperatures $\geq 0^{\circ}\text{C}$ (Mandel, Byrt and Ada,1969; Ada,1970; Dwyer and Mackay,1972; Bankhurst and Wilson,1971).
- (b) ABC which bind one antigen do not bind unrelated antigens (Naor and Sulitzeanu,1969; Byrt and Ada,1969; Ada and Cooper,1971; Laskov,1968).
- (c) Immunized animals show an increased incidence of cells binding the homologous or related antigens, but not those binding unrelated antigens (Naor and Sulitzeanu,1969; Humphrey and Keller,1970; Ada and Cooper,1971; Laskov,1968).
- (d) Essentially all ABC are lymphocytes. Macrophages bind antigen specifically under some circumstances but this has been shown to involve cytophilic antibodies (Nossal and Ada,1971).
- (e) Some anti-immunoglobulin reagents inhibit the binding of antigen to ABC (considered later).
- (f) A hierarchy of ABC is usually observed, *i.e.* some cells appear to bind more antigen than others (Ada,1970). This suggests that any two ABC binding the same antigen may have (i) dissimilar numbers of the same receptor or (ii) similar numbers of different receptors. The latter

is supported by the changes in antibody heterogeneity and affinity observed during humoral responses to various antigens (Werblin and Siskind, 1972).

Quantitatively, the results obtained using macroscopic or soluble antigens to detect ABC will depend on the conditions used to label cells and on the criteria used in each case to identify ABC. In mice immune to fowl gamma globulin (FGG), Bankhurst and Wilson (1971) found that 0.8 % of splenic lymphoid cells were labeled (in autoradiographs) by ^{125}I -labeled FGG, whereas only 0.16 % formed rosettes with FGG-coated RBC. Of these RFC, only 59 % were labeled. Thus, in this study at least, it is clear that each method detected different although overlapping populations of ABC. It was suggested by these authors that this differential detection of RFC and ABC is due to the fact that a RFC has only to bind 10-15 erythrocytes in order to be detected whilst an ABC may have to bind at least 4,000 molecules of radiolabeled antigen (Byrt and Ada, 1969), *i.e.* cells with very low receptor densities may be detected as RFC provided their receptors are of sufficient affinity to remain bound to the RBC. For example, if an ABC must be linked to an erythrocyte by at least 100 receptors (an arbitrary figure) for the two to remain associated, then a cell possessing 2,000 receptors of high affinity might, with only 50 % of its receptors saturated, bind up to 10 RBC and be detected as a RFC, but may not be detected as an ABC by autoradiography. In contrast, a cell with many receptors (say 50,000) of low affinity may bind sufficient radioactive antigen to be labeled but may not be able to retain bound RBC.

1.1.2 Immunocompetence of ABC

At least some ABC appear necessary for the full expression of both humoral and/or cell-mediated immunity, as evidenced by radiolabeled antigen-mediated "suicide" and by specific depletion of ABC on columns of antigen-coated beads.

Prolonged exposure of lymphoid cells to ^{125}I -labeled antigen of high specific radioactivity decreases their ability to transfer antibody responsiveness to lethally-irradiated recipients (Ada and Byrt, 1969; Basten, Miller, Warner and Pye, 1971; Roelants and Askonas, 1971; Unanue, 1971a,b). Mice injected with highly radioactive antigen also fail to elicit a humoral

response against the same antigen upon subsequent challenge (Humphrey and Keller, 1970). Antibody responsiveness to serologically unrelated antigens is unaffected and treatment of cells with either unlabeled or nonradioactive ^{127}I -labeled antigen results in normal antibody responsiveness. Both T and B lymphocytes can be inactivated. Exposure to ^{125}I -labeled antigen specifically diminishes the ability of T cells to (a) co-operate in a humoral response (Basten et al., 1971; Roelants and Askonas, 1971) or to (b) transfer cell-mediated immunity (Cooper and Ada, 1972), and of B cells to develop into antibody-forming cells (Basten et al., 1971). It is thought that only those cells which bind the most antigen are inactivated (Ada, 1970) although the proportion of ABC which these cells constitute is at present unknown.

The functional importance of ABC is further demonstrated by the reduced immunocompetence of lymphoid cell populations after passage through columns of antigen-coated beads (Wigzell and Anderson, 1969; Abdou and Richter, 1969; Wigzell, 1970; Henry, Kimura and Wofsy, 1972). No loss of immunocompetence is observed if the beads are coated with unrelated antigens, or if excess antigen (the same as that on the beads) is included in the medium used to wash the cells through the column.

1.1.3 Incidence of T and B ABC

What proportion of ABC are thymus-derived? Estimates have relied on changes in the incidence of ABC effected by anti- θ serum (Raff, 1969) and complement, or identification of thymus-derived ABC (T ABC) by labeling with fluorescent anti- θ antibody. The validity of these methods requires that T ABC contain θ antigen in amounts detectable by immunofluorescence or by anti- θ -induced cytolysis, and also that anti- θ sera contain no other antibodies which might affect nonthymus-derived ABC.

The proportion of ABC identified as thymus-derived may also depend on the method used to detect ABC. It was mentioned earlier that Bankhurst and Wilson (1971) detected fewer RFC using FGG-coated RBC than ABC using ^{125}I -labeled FGG. In the same study it was shown that after treatment with anti- θ serum and complement, the proportion of RFC which were labeled in autoradiographs increased from 59 % to 86 %. Thus there was a higher proportion of T cells amongst the unlabeled RFC than

amongst the labeled RFC. If this result is interpreted according to the hypothesis presented earlier (p. 5), then T ABC must possess fewer receptors than B ABC and most of the cells labeled by ^{125}I -labeled FGG would be B cells. Unfortunately the effect of anti- θ treatment on either labeled cells or RFC as separate populations was not reported.

Because of these factors, estimates of the proportion of T cells in ABC populations vary considerably. In addition, the proportion may change upon immunization if T or B ABC increase disproportionately in number.

In the spleens of unprimed mice, the estimated proportion of SRBC-specific RFC which are anti- θ sensitive ranges from < 20 % to 48 % (Greaves and Möller, 1970; Wilson and Miller, 1971; Ashman and Raff, 1973). Roelants (1972b), using *Maia squinado* haemocyanin and also the synthetic polypeptide 'TIGAL', estimated the proportion of ABC which were lysed by anti- θ serum and complement to be 20-30 % in spleens of unprimed mice. Most of these cells bound fewer molecules of antigen than did anti- θ -resistant ABC. In immune mice, however, θ -positive ABC (*i.e.* ABC labeled by fluorescent anti- θ antibody) bound as many antigen molecules as did θ -negative ABC (Roelants, Forni and Pernis, 1973). After immunization, both the incidence of ABC for the immunogen and the proportion of ABC identified as T cells are reported to increase (Bankhurst and Wilson, 1971; Wilson and Miller, 1971; Roelants, 1972b).

It is significant to this discussion that the frequency of ABC detected by autoradiography in spleens from unimmunized normal, neonatally-thymectomized, or congenitally-athymic mice is similar (Dwyer, Mason, Warner and Mackay, 1971). As considered above, this technique selectively detects those ABC possessing the most receptors. Thus, if T cells possess fewer receptors than B cells such that T ABC are not readily detected by autoradiography, then the above result would be likely. The similar incidence of ABC in each type of mouse strongly suggests that this technique does detect mainly nonthymus-derived ABC.

Finally, it should be noted that the incidence of T ABC is antigen-dependent, *i.e.* in spleens of mice immune to the homologous antigen, the proportion of RFC lysed by anti- θ

serum and complement was 0 % for *E. coli* lipopolysaccharide (a thymus-independent antigen) and 31 % for sheep RBC or the hapten 4-hydroxy-3,5-dinitrophenyl acetic acid (NNP), both T-dependent antigens (Möller and Sjöberg, 1972). It is not known whether significant variation occurs between different T-dependent antigens.

1.1.4 Nature of ABC receptors for antigen

The binding of radiolabeled antigens to ABC from mice is significantly reduced by polyvalent anti-immunoglobulin sera (Byrt and Ada, 1969; Dwyer and Warner, 1971; Roelants, Forni and Pernis, 1973) and generally by anti-light or anti- μ but not by anti- γ -chain sera (Warner, Byrt and Ada, 1970; Roelants, Forni and Pernis, 1973). A similar situation is found in man (Dwyer and Mackay, 1970; 1972). However, anti- γ -chain sera are reported more effective than anti- μ against ABC from guinea pigs (Davie and Paul, 1971; Davie, Rosenthal and Paul, 1971) and in one instance in mice (Unanue, 1971a).

Anti-immunoglobulin reagents, particularly those directed against light (L) or μ heavy chains, also inhibit rosette formation (Greaves and Möller, 1970; Hogg and Greaves, 1972; Möller and Sjöberg, 1972; Ashman and Raff, 1973) and depletion of immunocompetent cells by antigen-coated beads (Wigzell, 1970; Rutishauser, Millette and Edelman, 1972). The bulk of ABC detected in these experiments are probably B cells (see above) and as anti-L or anti- μ -chain sera usually reduce the number of detectable ABC in the mouse and man by > 70 %, the receptor for antigen on B ABC is almost certainly closely associated with (if not) an immunoglobulin, probably IgM.

Several results indicate that the receptors on T ABC are similar to those of B cells. Firstly, θ -positive RFC are significantly suppressed by polyvalent anti-immunoglobulin sera (Ashman and Raff, 1973) and by anti- μ -chain sera but not by sera against other immunoglobulin heavy chains (Hogg and Greaves, 1972). Binding of radiolabeled antigens to θ -positive ABC is also significantly inhibited by polyvalent anti-Ig, anti- μ or anti-L-chain sera (Roelants, Forni and Pernis, 1973). Secondly anti-L-chain sera prevent the inactivation (by ^{125}I -labeled antigen) of immunocompetent B and T cells in mice (Basten et al., 1971; Cooper and Ada, 1972).

1.2 Lymphocyte surface-immunoglobulin

1.2.1 Methods of detection

Techniques for demonstrating cell surface-Ig all rely at some stage on the recognition of Ig molecules by anti-Ig antibodies. Many of these techniques measure the binding of anti-Ig reagents to live lymphocytes, either *directly* by immunofluorescence, immunautoradiography, or rosette formation (using anti-Ig-coated RBC) or *indirectly* by measuring secondary effects such as lymphocyte transformation, complement-mediated cytotoxicity, altered electrophoretic mobility, suppression of immune responses, or reduction of antigen binding. Alternatively, antibody consumption tests which measure the capacity of lymphocytes to neutralize anti-Ig antibody activity (assayed by radioimmunoassay or by passive haemagglutination) can be used. More recently, surface-Igs have been radiolabeled (biosynthetically, or using lactoperoxidase) and extracted from cells prior to their identification by serological and chemical means. Individual cells which can be shown by these methods to have large quantities of Ig on their surface will be hereafter abbreviated as "Ig⁺", whereas cells which cannot be readily shown to bear surface-Ig will be termed "Ig⁻".

1.2.2 Nature of Ig⁺ and Ig⁻ lymphocytes

Studies showing that anti-Ig antibodies are mitogenic for lymphocytes (reviews: Sell, 1970; Greaves, 1970), alter their electrophoretic mobility (Bert, Massaro, Di Cossano and Maja, 1969), and inhibit their antigen-binding capacity (above) provided substantial indirect evidence for the existence of lymphocyte surface-Ig.

In 1970, Raff *et al.* reported that mice contained two distinct populations of peripheral lymphocytes, one thymus-derived and labeled by fluorescent anti- θ antibody, the other thymus-independent and labeled by fluorescent or ¹²⁵I-labeled anti-mouse-Ig antibody (Raff, 1970; Raff, Sternberg and Taylor, 1970). This was confirmed in adult-thymectomized, lethally-irradiated CBA mice reconstituted with syngeneic bone marrow cells and, in some cases, 4 weeks later with syngeneic or semisyngeneic F₁ thymocytes (Unanue, Grey, Rabellino, Campbell and Schmidtke, 1971). Examination of the spleens of these mice

12 weeks after irradiation indicated that essentially all Ig^- lymphocytes were thymus-derived and that the majority of Ig^+ lymphocytes were B cells. This conclusion is also supported by the high incidence of Ig^+ lymphocytes in thoracic duct lymph of congenitally-athymic mice (94 %) compared to that of normal mice (24 %) (Bankhurst and Warner, 1972), and by the selective cytolysis of B lymphocytes by anti- κ -chain serum (Miller, Sprent, Basten and Warner, 1972; Takahashi, Old, McIntire and Boyse, 1971).

The incidence of Ig^+ lymphocytes in various tissues of mice is shown in Table 1.1. In chickens, Ig^+ lymphocytes are bursa-derived (Rabellino and Grey, 1971) and constitute nearly 100 % of bursal lymphocytes in day-old birds (Kincade, Lawton and Cooper, 1971). Thus in these two species most and possibly all mature B lymphocytes are Ig^+ . In contrast, thymocytes and T lymphocytes are Ig^- , i.e. they do not possess readily-detectable surface-Ig.

Ig^+ lymphocytes are also found in the *rabbit* (Pernis, Forni and Amante, 1970, 1971; Davie, Paul, Mage and Goldman, 1971), *rat* (Avrameas and Guilbert, 1971; Unanue, Perkins and Karnovsky, 1972a,b), *man* (Coombs, Feinstein and Wilson, 1969; Biberfeld, Biberfeld and Perlman, 1971; Fröhland and Natvig, 1971; Heller, Bhoopalam, Yakulis and Costea, 1971; Pernis, Forni and Amante, 1971; Grey, Rabellino and Pirofsky, 1971; Johansson and Klein, 1970), *sheep* (Chapter 5) and *amphibia* (Du Pasquier, Weiss and Loor, 1972). The incidence of Ig^+ cells in lymphoid tissues in these animals is similar to that found in mice, being lowest in thymus and highest in spleen.

1.2.3 Class and origin of surface-immunoglobulins

The possibility that surface-Igs may be humoral Igs adsorbed by the cells on which they are detected is of particular concern in studies of lymphocyte surface-Ig, as mouse B lymphocytes have been shown to possess on their surfaces receptors which bind the Fc moiety of Ig molecules (Basten, Miller, Sprent and Pye, 1972; Paraskevas, Lee, Orr and Israels, 1972). Ig bound in this manner is termed 'cytophilic'. To determine whether cytophilic Igs contribute significantly to the Ig present on Ig^+ lymphocytes, some workers have utilized the phenomenon of allelic exclusion. Thus, it was

Table 1.1

Incidence^{a)} in mouse tissues of lymphocytes bearing θ antigen
or Ig determinants on their surface membranes

MOUSE STATUS	TISSUE	ANTI- θ		ANTI-IMMUNOGLOBULIN			
		Fluor. ¹⁾	Lysis ²⁾	Polyvalent ³⁾		Anti- κ ⁴⁾	Anti- μ ⁴⁾
				Fluor.	ARG	Lysis	Lysis
NORMAL	Thymus	95-100	100	0	0.2	0	0
	Bone marrow	-	0	-	15	30	20
	Blood	-	71	-	-	-	-
NORMAL NUDE ^{b)} , 5)	Thoracic	85-90	88	-	14	15	10
	duct lymph	-	-	-	94	-	-
NORMAL ATXBM ^{c)} , 6)	Peripheral	78-85	61	19	17	40	30
	lymph	-	-	71-78	-	-	-
	ATXBM + thymus ⁶⁾ node	-	-	32-35	-	-	-
NORMAL ATXBM ⁶⁾	Spleen	30-50	31	35	41	50	40
	"	-	-	85-100	-	-	-
	ATXBM + thymus ⁶⁾ "	-	-	53-55	-	-	-

a) Percentage of lymphocytes binding antibody as detected by immunofluorescence (Fluor), immunoautoradiography (ARG), or complement-mediated cytotoxicity (Lysis).

b) Nude mice: congenitally-athymic.

c) ATXBM: adult-thymectomized, lethally-irradiated, bone marrow-reconstituted mice.

Data taken from: (1) Raff, 1970; (2) Raff, 1971; (3) Raff, Sternberg and Taylor, 1970;
 (4) Takahashi, Old, McIntire and Boyse, 1971; (5) Bankhurst and Warner,
 1972; (6) Unanue, Grey, Rabellino, Campbell and Schmidtke, 1971.

found that almost all Ig⁺ rabbit lymphocytes exhibit allelic exclusion for both *a* and *b* locus allotypes, indicating that these cells almost certainly synthesized their own surface-Ig (Pernis, Forni and Amante, 1970, 1971; Davie, Paul, Mage and Goldman, 1971). Furthermore, the only class of surface-Ig detected was IgM, although both IgM and IgG were demonstrated intracellularly (Pernis, Forni and Amante, 1970, 1971).

Unfortunately, the contribution of cytophilic Ig has not been evaluated in many studies and it is therefore difficult to ascertain the origin, and consequently the significance, of the various classes of surface-Ig which are reported. The presence on the surface of lymphocytes of determinants belonging to more than one Ig class was first indicated by the mitogenic properties of antibodies directed against different Ig light and heavy chains (Sell, 1970; Sell, Lowe and Gell, 1970; Greaves, 1970), and determinants characteristic of κ and λ light chains and of μ , γ , α and (in humans) δ heavy chains have since been demonstrated by direct labeling of lymphocytes with fluorescent or radiolabeled antibodies (Pernis, Forni and Amante, 1971; Grey, Rabellino and Pirofsky, 1971; Fröhland and Natvig, 1971; Jones, Torrigiani and Roitt, 1971; Bankhurst and Warner, 1972; Rowe, Hug, Faulk, McGormick and Gerber, 1973).

In some cases single lymphocytes are reported to bear surface-Ig of only one class, although different cells may bear different classes (Jones, Torrigiani and Roitt, 1971; Rabellino, Colon, Grey and Unanue, 1971; Fröhland and Natvig, 1971; Grey, Rabellino and Pirofsky, 1971). This is usually judged by determining whether the total proportion of cells labeling for *all* heavy chains equals or exceeds that labeling for light chains. In other instances, single cells are found to possess surface-Igs of more than one class (Bankhurst and Warner, 1971; Nossal, Warner, Lewis and Sprent, 1972; Chapter 5). However, in nearly all of these studies, more lymphocytes are heavily labeled by antibodies directed against μ chains than by those directed against other Ig heavy chains. In many of these reports, some classes of surface-Ig may arise by cytophilic binding. The clarification of this point is important in determining whether a single lymphocyte is able to synthesize one or more classes of Ig, and particularly whether one or more

classes of Ig can function in the surface membrane as a receptor for antigen. Single cells which simultaneously synthesize more than one class of Ig (as evidenced by the demonstration of more than one Ig in their cytoplasm) do occur, but are infrequent in populations of normal or immune lymphoid cells (Nossal *et al.*, 1963; Costea *et al.*, 1967; Takahashi *et al.*, 1968; Pernis, Forni and Amante, 1971).

1.2.4 Lymphocyte surface-IgM

In every vertebrate so far examined, antibodies specific for light or μ heavy chains bind in greater amounts to a higher proportion of lymphocytes than do antibodies directed against other Ig heavy chains. Furthermore the only Ig determinants detected by immunofluorescence on the surface of blood lymphocytes (obtained from lymphocytic leukemia patients who each possessed a greater-than-normal proportion of Ig⁺ lymphocytes and for whom these cells were Ig class-tested) were those of μ heavy chains (29/34 cases; no heavy chain determinants were detected in the other 5), κ light chains (26/34) and λ light chains (8/34) (Johansson and Klein, 1970; Klein, Eskeland, Inoue, Strom and Johansson, 1970; Grey, Rabellino and Pirofsky, 1971; Pernis, Forni and Amante, 1971). The IgM⁺ cells from several of these patients, none of whom exhibited monoclonal hypergammaglobulinemia, could not be shown to secrete IgM (Johansson and Klein, 1970).

Fractionation of the cells from one patient indicated that most of the IgM determinants were situated in the plasma membrane (Eskeland, Klein, Inoue and Johansson, 1971). Using fluorescent anti- μ -chain antibody to label cells, it was found that 90-100 % of blood lymphocytes from this patient were IgM⁺ (Johansson and Klein, 1970). Furthermore, by using intact cells to neutralize anti- μ -chain antibody (assayed by agglutination of IgM-coated RBC), it was estimated that 10^6 lymphocytes had the equivalent of 25 ng of IgM on their surfaces (Klein *et al.*, 1970; Eskeland *et al.*, 1971). All the membrane IgM solubilized during subcellular fractionation was shown by sedimentation centrifugation to be present as a 7S molecule, in contrast to serum IgM which is a 19S molecule (Eskeland *et al.*, 1971). Each cell was estimated to have approximately 80,000 7S IgM molecules exposed on its surface. Similar values (50,000-150,000 7S molecules/Ig⁺ cell)

have been calculated for mouse lymphocytes (Rabellino, Colon, Grey and Unanue, 1971; Grey, Colon, Campbell and Rabellino, 1972; Unanue, Grey, Rabellino, Campbell and Schmidtke, 1971).

Lactoperoxidase-catalyzed radioiodination of intact lymphocytes has also been used to reveal the presence of IgM on the cell surface. This procedure is reported to label only surface membrane components (Marchalonis, Cone and Santer, 1971; Baur, Schenkein and Uhr, 1972). After washing, the labeled cells are disrupted, carrier Ig is added and Igs are precipitated from the cell extracts with Ig-specific antisera. The washed precipitates are redissolved in dissociating solvents and examined for radiolabeled components by chromatography and/or gel electrophoresis.

Thus, light and μ heavy chains have been isolated from the surface of human lymphoma cells (Baur, Vitetta, Sherr, Schenkein and Uhr, 1971) and mouse spleen cells (Baur *et al.*, 1971; Vitetta, Baur and Uhr, 1971; Marchalonis, Cone and Atwell, 1972; Grey, Kubo and Cerottini, 1972). Little labeled γ chain was isolated from mouse spleen cells (Vitetta, Baur and Uhr, 1971) although γ and light chains could be isolated from the surface of IgG-secreting mouse myeloma cells (Baur, Schenkein and Uhr, 1972). These results indicate that IgM is the major Ig on the surface of mouse spleen cells. Furthermore, surface-IgM isolated without reduction from mouse spleen cells is virtually all 7S (Vitetta, Baur and Uhr, 1971; Vitetta and Uhr, 1972a,b), as is the IgM on the surface of human leukemia cells (Eskeland *et al.*, 1971).

The use of [^3H]-tyrosine to biosynthetically label cell proteins, and ^{125}I to label surface-proteins, has indicated that spleen cell populations from unimmunized mice (a) synthesize and secrete both IgM (mainly 7S, but some 19S) and IgG and (b) incorporate a proportion of the 7S IgM but virtually no IgG into their surface membranes (Vitetta and Uhr, 1972a,b). Similarly, Parkhouse (1973) has reported that although IgG and IgM are synthesized by mouse lymph node cells (2/1, wt/wt), IgG only is secreted and 30-35 % of the cells possess surface-IgM, as indicated by labeling with fluorescent anti- μ -chain antibody.

In view of these reports, it is not surprising that

7S IgM is the only labeled Ig released by radioiodinated spleen cells at 37°C (Vitetta and Uhr, 1972a,b). A variety of labeled membrane components seem to be released (Cone, Marchalonis and Rolley, 1971) and < 5 % of the released radioactivity can be precipitated with anti-Ig sera. The release of these components is temperature-dependent, but there is uncertainty as to whether cellular metabolism is involved (Cone, Marchalonis and Rolley, 1971; Vitetta and Uhr, 1972a,b). Surface-IgM may be released associated noncovalently with other membrane components (Vitetta and Uhr, 1972a,b).

1.2.5 Lymphocyte membrane modulation ("patching" and "capping")

Numerous investigators using fluorescent anti-Ig antibodies to label intact lymphocytes often noted that cells did not always label in a uniform manner. In some cases, the cells were labeled uniformly, giving a ring-like effect; in others, a patchy distribution was observed; and in yet others the label was concentrated at one pole of the cell, in the form of a "cap". Membrane modulation is not unique to anti-Ig reagents but may be induced by incubating lymphocytes (and other cell types, e.g. Leonard, 1973) with multivalent antibodies directed against cell-surface components or with certain agglutinins (Taylor et al., 1971; Loor, Forni and Pernis, 1972; Unanue, Perkins and Karnovsky, 1972a,b; de Petris and Raff, 1972, 1973; Elson, Singh and Taylor, 1973; Hütteroth, Cleve and Litwin, 1973).

The rapidity at which cap formation and subsequent endocytosis occur depends on a number of factors and the process may be stopped artificially at each of the visually-distinct stages. Cap formation in mouse splenic lymphocytes results from two independent processes (Taylor et al., 1971; Loor, Forni and Pernis, 1972):

(a) Patch formation

This is thought to involve diffusion of membrane components in the plane of the membrane and their cross-linkage via divalent antibodies. Monovalent rabbit (anti-mouse-Ig) Fab fragments produce uniform labeling but are unable to initiate spot formation without the addition of divalent anti-rabbit-Ig

antibody, which cross-links them ("piggy-back" effect). These observations correlate with the ability of divalent, but not monovalent Fab, antibodies to stimulate transformation of human or rabbit lymphocytes (Woodruff, Reid and James, 1967; Fanger, Hart, Wells and Nisonoff, 1970). However, anti-Ig capping can occur without causing stimulation (Elson, Singh and Taylor, 1973).

Conditions that inhibit diffusion of membrane components should inhibit patch formation. Thus the rate of patch formation is markedly decreased by (a) low temperatures ($< 20^{\circ}\text{C}$), which possibly increase the viscosity of membrane phospholipids, and (b) Concanavalin A or high concentrations of sugars such as galactose or mannose, which may interact with and restrict the freedom of membrane glycoproteins (Loor, Forni and Pernis, 1972). Antimetabolites, such as sodium azide, 2,4-dinitrophenol, or inhibitors of protein synthesis have little to no effect (Taylor *et al.*, 1971; Loor, Forni and Pernis, 1972; Hütteroth, Cleve and Litwin, 1973). Excess antibody can inhibit patch formation by saturating membrane components so that they cannot be crosslinked (Taylor *et al.*, 1971). The efficiency of patch formation presumably depends on the antibody concentration and the density, size and mobility of the membrane antigens. Mono-determinant surface-antigens should not mediate patch formation except via a "piggy-back" antibody.

(b) Cap formation

Capping, which involves the aggregation of patches, does not occur via simple diffusion but as a result of cell motility. It is markedly temperature dependent in the range $15-25^{\circ}\text{C}$ and is completely inhibited by antimetabolites, such as sodium azide or 2,4-dinitrophenol which prevent energy-production (Taylor *et al.*, 1971; Loor, Forni and Pernis, 1972; de Petris and Raff, 1973). It is thought that during cell movement mobile membrane components flow forward whilst immobilized regions, such as "patches", accumulate and cross-link further at the tail, or uropod, of the cell to form a "cap".

Endocytosis, which begins during cap formation, may be partial or complete, depending on the nature of the aggregated surface-component (Unanue, Perkins and Karnovsky,

1972a,b). Surface-Ig, once capped, is rapidly and completely endocytosed (Loor, Forni and Pernis, 1972; Wilson, Nossal and Lewis, 1972; Unanue, Perkins and Karnovsky, 1972a,b).

Incubation of mouse spleen cells in the presence of, or after pulse-labeling with anti-Ig antibody has indicated that the cell-bound antibody disappears from the cell surface within 30-60 min. at 37°C (Loor, Forni and Pernis, 1972; Elson, Singh and Taylor, 1973; Hütteroth, Cleve and Litwin, 1973). A similar disappearance of surface-Ig occurs during incubation of spleen cells with *E. coli* lipopolysaccharide (Melchers and Andersson, 1973), and with antigen (Diener and Paetkau, 1972; Raff, Feldman and de Petris, 1973). Replacement of surface-Ig by the lymphocytes requires approximately 5-20 h (Loor, Forni and Pernis, 1972; Elson, Singh and Taylor, 1973; Hütteroth, Cleve and Litwin, 1973; Melchers and Andersson, 1973). These experiments strongly suggest that IgM⁺ lymphocytes do synthesize their own surface Ig.

1.2.6 Synthesis of IgM by B lymphocytes

Recently Melchers and Andersson (1973) have utilized the capping phenomenon to aggregate surface-Ig on spleen cells of congenitally-athymic (nu/nu) mice so that the Ig is insoluble in detergent extracts of the cells. By pulse-labeling cells with [³H]-leucine, -mannose, -galactose or -fucose and 'chasing' in nonradioactive medium for various periods before capping all the surface-Ig and dissolving the cells with Nonidet P-40, these investigators were able to measure the synthesis of IgM and its secretion into the medium or its incorporation into the surface membrane. Their findings are summarized below:

(a) Unstimulated nu/nu mouse spleen cells

(i) Populations of unstimulated B cells synthesize small amounts of IgM but do not synthesize IgG in detectable quantities.

(ii) A proportion of newly-synthesized intracellular 7S IgM (IgMs) sequentially acquires carbohydrate residues during its passage to the cell surface where it is polymerized and immediately secreted as 19S IgM. The synthesis and secretion of this IgM has a half-life of 4-8 h.

(iii) The newly-synthesized IgMs, which is not rapidly-

secreted acquires only a fraction of the carbohydrate residues found in secreted 19S IgM and is incorporated into the plasma membrane as surface-IgM. This IgM is not polymerized, contains almost no galactose, fucose or N-acetylneuraminic acid residues, and has an apparent half-life of 30-80 h.

These processes are represented diagrammatically in Fig 1.1.

(b) Lipopolysaccharide-stimulated nu/nu mouse spleen cells

(i) The surface-IgM of B cells cultured *in vitro* in the presence of mitogenic concentrations of *E. coli* lipopolysaccharide (LPS) becomes aggregated and disappears from the cell surface during the first 30 min. of culture. This IgM, which presumably caps and becomes endocytosed by the cells, is slowly digested by proteases. Such cells remain devoid of surface-IgM until 25-30 h of LPS stimulation.

(ii) After 10-15 h stimulation with LPS, total cellular protein and IgM synthesis increases. IgMs molecules reappear on the lymphocyte surface after 25-30 h stimulation, in greater numbers than were originally present. A similar phenomenon is observed after capping surface-Ig with either antigen (Diener and Paetkau, 1972) or anti-Ig antibody (Loor, Forni and Pernis, 1972). As judged by immunofluorescence, more than half the cells have increased quantities of surface-IgM.

(iii) Simultaneous with the reappearance of surface-IgM, secretion of 19S IgM (half-life, 4-8 h) is begun on a scale greatly exceeding that of unstimulated cells. The secretion of large numbers of 19S IgM molecules is thought to be responsible for the increased numbers of IgMs molecules on the cell surfaces. Pokeweed mitogen has also been observed to selectively increase IgM synthesis in mouse B lymphocytes (Parkhouse, Janossy and Greaves, 1972).

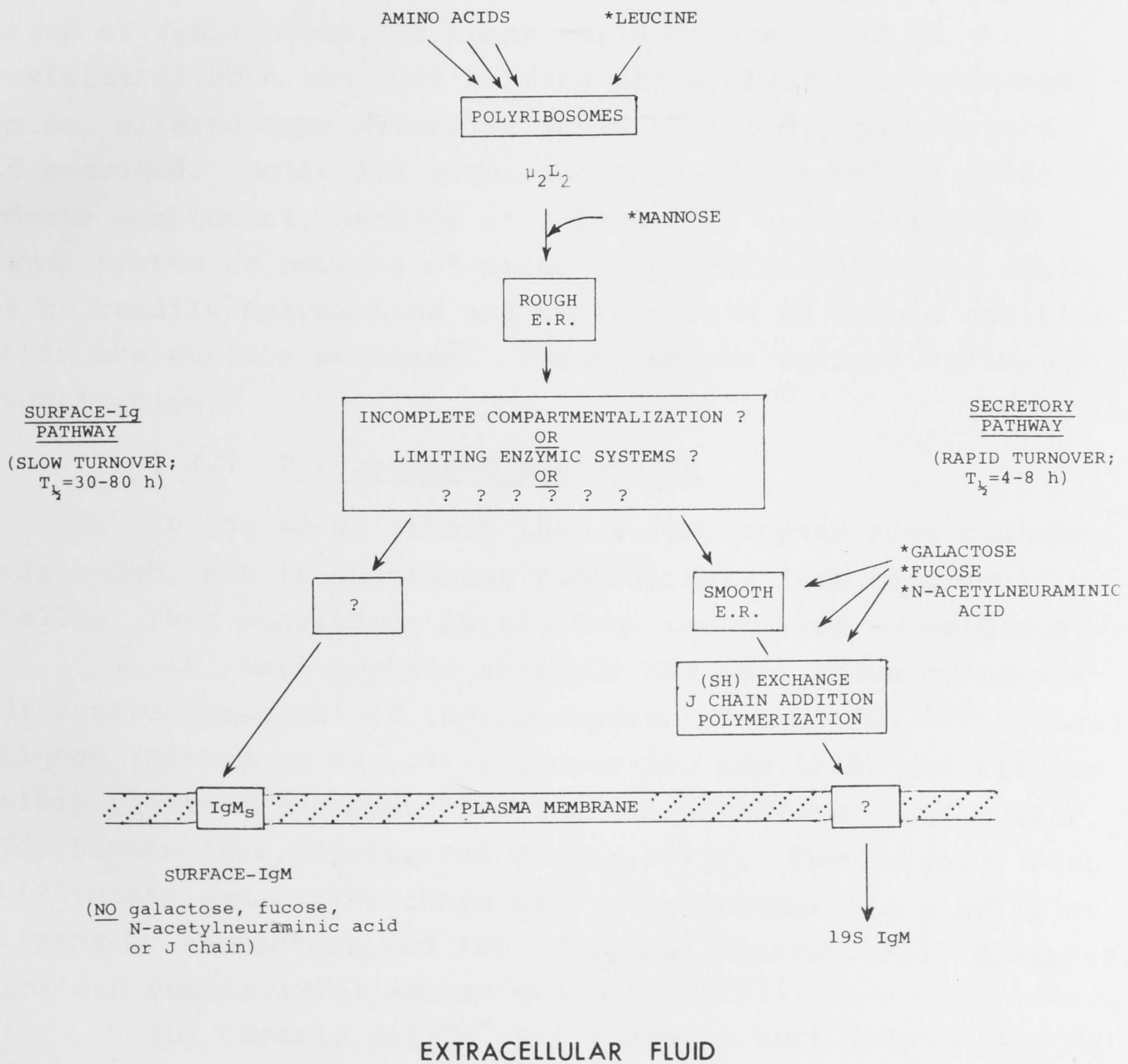
Surface-IgM thus differs from secreted 19S IgM in two important ways. Firstly surface-IgM lacks the semiterminal galactose and terminal fucose and N-acetylneuraminic acid carbohydrate residues found in all secreted 19S IgM. Secondly surface-IgM molecules are 7S IgM subunits (IgM_s).

It has been suggested (Melchers and Andersson, 1973) that the addition of the missing carbohydrate residues to IgMs

Figure 1.1

Proposed pathways of synthesis and secretion of IgM
in B lymphocytes

(constructed from data of Melchers and Andersson, 1973, and Della Corte and Parkhouse, 1973)



molecules may alter their conformation and allow polymerization and secretion. However IgMs molecules lacking a full carbohydrate complement can be polymerized *in vitro* by the covalent addition of a joining (J) chain in the presence of a disulphide-exchange system (Della Corte and Parkhouse, 1973). Nevertheless, the addition of these residues may increase the affinity of the IgMs molecule for the disulphide-exchange system so that the IgM is rapidly and efficiently polymerized. Furthermore, IgMs molecules are probably very hydrophobic, considering their stability in the plasma membrane. It is possible that the addition of carbohydrate residues may decrease the hydrophobic nature of IgMs. Thus, if these residues are added in the proximity of both the cell surface and a disulphide-exchange system, altered IgMs molecules would be rapidly polymerized and secreted. Molecules acquiring only an incomplete carbohydrate complement, perhaps as a result of a rate-limiting enzyme system or because of prior compartmentalization, would not be readily polymerized and would remain as stable entities within the surface membrane. These aspects warrant further investigation.

1.2.7 T Lymphocyte surface-Ig

It now seems likely that T lymphocytes also contain surface-IgM, but in quantities considerably less than found on B cells. This conclusion is based on the following evidence:

(a) Anti-L-chain antibody has been shown to prevent radioactive 'suicide' of immunocompetent T cells by ^{125}I -labeled antigens (Basten *et al.*, 1971; Cooper and Ada, 1972) and also to inhibit graft-versus-host reactions in mice (Mason and Warner, 1970; Riethmüller, Rieber and Seeger, 1971). Furthermore, both anti-L-chain and anti- μ -chain sera can prevent the binding of antigens by thymus-derived ABC (Hogg and Greaves, 1972; Roelants, Forni and Pernis, 1973; Ashman and Raff, 1973).

(b) Nearly all Ig^- rat thoracic duct lymphocytes can specifically bind ^{125}I -labeled anti-rat-Fab antibodies, but in quantities (200-3,000 molecules/cell) considerably less than bound by Ig^+ (B?) lymphocytes (20,000-150,000 molecules/cell) (Jensenius and Williams, 1973a). A similar phenomenon has been observed for mouse spleen cells (Nossal, Warner, Lewis and Sprent, 1972).

(c) Radioactive Ig possessing antibody activity is released at 37°C by radioiodinated thymus-derived mouse thoracic duct lymphocytes activated against histocompatibility antigens (Cone, Sprent and Marchalonis, 1972).

These findings indicate that the antigen receptors on both immunocompetent and antigen-binding T lymphocytes are closely associated with both μ and light Ig chains, and also that most peripheral T lymphocytes have small amounts of μ and light chains, probably as IgM, on their surfaces. At present, the possibility that this T lymphocyte surface IgM is cytophilic cannot be excluded (Webb and Cooper, 1973).

There have been numerous reports concerning surface-Ig on thymus cells. These cells have attracted attention mainly for their unlikely contamination by Ig⁺ (B) lymphocytes, although < 5 % are immunocompetent (Blomgren and Andersson, 1971) and the majority may not be typical of mature T lymphocytes. A small proportion (< 3 %) of mouse thymocytes are labeled heavily by anti-Ig antibodies, but these cells are reported to be not typical of thymocytes, B lymphocytes or plasma cells (Bankhurst and Warner, 1971; Hämmerling and Rajewsky, 1971; Perkins, Karnovsky and Unanue, 1972; Kirov and Ada, to be published) and are deficient in θ and TL (thymocyte) antigens but positive for surface-Ig and the plasma cell alloantigen PC.1 (Vitetta, Uhr and Boyse, 1973). In addition, the Ig synthesized and secreted by these Ig⁺ cells (IgG and 19S IgM) accounts completely for the Ig produced by whole thymus (Vitetta, Uhr and Boyse, 1973) and may also account for the isolation of μ and light Ig chains from radioiodinated thymocytes (Marchalonis, Atwell and Cone, 1972; Marchalonis, Cone and Atwell, 1972). Others, using similar techniques, have not detected surface-Ig on thymocyte populations (Grey, Kubo and Cerottini, 1972; Vitetta, Bianco, Nussenzweig and Uhr, 1972). Radioimmunoassays have indicated that mouse thymus cell populations contain very little surface or total Ig compared to spleen cell populations, (Unanue, Grey, Rabellino, Campbell and Schmidtke, 1971; Grey, Colon, Campbell and Rabellino, 1972; Grey, Kubo and Cerottini, 1972; Jensenius and Williams, 1973b).

1.3 Aims and outlines of thesis

This thesis describes studies aimed at elucidating the identity of antigen-receptors on B lymphocytes. Isolation and direct characterization of these receptors for antigen is necessary to determine unequivocally whether they are immunoglobulin and if so, whether and in what way they differ from humoral immunoglobulins. It is also particularly important to determine whether the receptors interact with any membrane component(s) which might cause the lymphocyte to be stimulated, perhaps by the activation of an enzymic process, after the receptors have bound antigen.

The problem was approached initially by isolating the lymphocyte plasma membrane in a state free of intracellular material, especially Ig. Once this was achieved it was intended to fractionate the membrane into its various components and to characterize them. Thus, it was hoped to identify the receptors and any component(s) which might be associated with them.

In order to distinguish membrane components from intracellular material which could adsorb to the membrane during its purification, a method was developed for labeling, with radioactive iodide, the plasma membranes of intact cells (Chapter 3).

The results of studies on sheep lymphocyte plasma membranes are described in Chapter 4. Sheep lymphocytes were chosen for this work because they were available in pure form and in large numbers. The membranes were fragmented under a variety of conditions, and using a number of membrane markers their distribution in density gradients was investigated. One finding of this study was that the detergent Nonidet P-40 can be used to dissolve the plasma membrane without dissociating membrane-bound antibodies from the surface antigens to which they are attached. This use of NP40 was subsequently extended to study the complexes formed between anti-Ig antibodies and lymphocyte surface-Ig (Chapter 6).

During these investigations it was discovered that anti-Ig antibodies bound in considerable amounts to intact sheep lymphocytes. Because surface-Ig is likely to be the receptor for antigen (see 1.1.4), this phenomenon was investigated in more detail. Thus, it was shown that a

proportion (20-30 %) of sheep lymph cells were labeled by anti-L, anti- μ , and (to a lesser extent) by anti- γ -chain antibodies (Chapter 5). Furthermore, by labeling the lymphocytes with anti-Ig antibodies and then dissolving them in NP40 it was found that IgM was present on the cell surface in both 7S and 19S forms (Chapter 6). The fate of the (surface-Ig/anti-Ig) complexes during incubation of labeled cells at 37°C was also determined (Chapter 6).

Thus, as had been found in lymphocyte populations of other vertebrates, a proportion of sheep lymphocytes possessed large quantities of Ig on their surface. Were these molecules the "true" receptors for antigen? Experiments were initiated with the aim of determining this, and preliminary studies investigating the ability of anti-Ig antibody to inhibit the *net* binding of antigens to populations of mouse spleen, lymph node and sheep lymph cells are described in Chapter 7. It is hoped that an extension of this approach will enable the receptors for a particular antigen to be isolated from a population of lymphocytes and identified.

1.4 Communications

Some of the work contained in this thesis has been presented at meetings and has been published, or is to be submitted for publication, as follows:

1. Ey, P.L. (1971). Immunoglobulins on the sheep lymphocyte surface. Abstr. Ann. Meet. Aust. Soc. Immunol., Melbourne, p. 7.
2. Ey, P.L. (1973). Immunoglobulins on the surface of sheep lymphocytes. I. Class and cellular distribution. Eur. J. Immunol. 3 : 37.
3. Ey, P.L. (1973). Immunoglobulins on the surface of sheep lymphocytes. II. Class, size and fate during incubation of lymphocytes at 37°C. Eur. J. Immunol. (in press).
4. Ey, P.L. A nonenzymic method for selective radioiodination of the plasma membranes of intact red blood cells. (in preparation).
5. Ada, G.L. and Ey, P.L. Lymphocyte receptors for antigen in "The Antigens", M. Sela (Ed.), Academic Press. Vol. 4 (in preparation).

2.1 General

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- 2.1.5 Immunoelectrophoresis
- 2.1.6 Radioassay
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- 2.1.8 Radioimmunoassay
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- 2.1.10 Antigens
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Chapter 2

2.2 Immunoglobulins, antisera and antibodies

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- 2.2.2 Reduction of sheep IgG: Separation of γ and δ chains
- 2.2.3 Sheep IgM
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- 2.2.6 Rabbit antisera and antibodies
- 2.2.7 Purity of sheep IgG and specificity of rabbit anti-sheep-Ig sera
- 2.2.8 Rabbit anti-sheep-Ig Fab fragments: Preparation and specificity

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- 2.2.7 Purity of sheep Igs and specificity of rabbit anti-sheep-Ig sera
- 2.2.8 Rabbit anti-sheep-Ig Fab fragments: Preparation and specificity

2.1 General

2.1.1 Media and reagents

The following solutions were prepared using double-distilled water by the Department of Microbiology Media Service, and stored at 4°C before use. *Saline*: 0.9 % (w/v) NaCl dispensed in 150 ml or 500 ml aliquots and autoclaved at 112°C for 15 min. *Alsever's solution*: NaCl (21 g), D-glucose (102.5 g), sodium citrate dihydrate (40 g) and citric acid (4 g) dissolved in water to a final volume of 5 litres. The solution was adjusted to pH 6.1 with 10 % citric acid, dispensed in 100 ml aliquots and autoclaved at 112°C for 20 min. *Dulbecco's balanced salt solution* (DBSS) was made by combining three solutions, each prepared separately (Dulbecco and Vogt, 1954): Phosphate-buffered saline (PBS) contained NaCl (8.0 g), KCl (0.2 g), Na₂HPO₄ (1.15 g), KH₂PO₄ (0.2 g) and water to 800 ml. It had a pH of 7.4, was dispensed in 400 ml aliquots and autoclaved at 121°C for 20 min; PBS2 consisted of CaCl₂ (0.1 % w/v) in 50 ml aliquots; PBS3 was MgCl₂·6H₂O (0.1 % w/v) in 50 ml aliquots. Both PBS2 and PBS3 were autoclaved at 112°C for 15 min. DBSS (500 ml) was prepared by combining PBS solutions 1, 2 and 3. *Eagle's minimal essential medium* (Cat. No. F-15, Grand Island Biological Co., N.Y.) filter-sterilized, was obtained from Dr. A.J. Cunningham. *Foetal calf serum* (FCS) was obtained from the Commonwealth Serum Laboratories (Parkville, Victoria).

Reagents were obtained as follows: Papain (2X crystallized, Cat. No. P-3125) and Tris(hydroxymethyl)amino-methane (Tris) ("Sigma 7-9") (Sigma Chemical Co.); 2-Mercaptoethanol (2ME) and N,N-Diethylaminoethyl(DEAE)-cellulose (Eastman Organic Chemicals, N.Y.); Ammonium sulphate (Mallinckrodt Chemical Works, U.S.A.); Nonidet P-40 (NP40; Shell Petroleum Co.). All other reagents were of laboratory or analytical grade.

2.1.2 Protein determination

Protein concentrations were usually estimated from absorbance at 280 nm (A_{280}). The extinction coefficient ($E_{280}^{1\%}$) used for IgG, L chains or γ chains was 14 and for IgM, 1 %

13, based on values published for rabbit and human Igs (Williams and Chase, 1968). Otherwise, where indicated in the text, protein was measured using Folin's reagent (Lowry *et al.*, 1951).

2.1.3 5'-Nucleotidase assay

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase; EC 3.1.3.5) activity was assayed by the method of Mitchell and Hawthorne (1965). Samples (0.4 ml) were mixed with 0.2 ml of assay solution to give final concentrations of 100 mM KCl, 10 mM MgCl₂, 10 mM L (+) NaK-tartrate, 50 mM Tris-HCl, pH 7.5 and 5 mM adenosine 5'-monophosphate (Calbiochem.). Blanks contained all but the nucleotide. Assay mixtures were incubated in a 37°C water bath and the reaction stopped by placing samples in ice and adding 0.2 ml iced trichloroacetic acid (1.5 M). The standard reaction time was 15 min. Inorganic phosphate released by the nucleotidase was measured by optical density at 820 nm (A₈₂₀) using the molybdate/ascorbic acid method of Ames and Dubin (1960).

2.1.4 Polyacrylamide gel electrophoresis

Two gel systems were employed, one using non-dissociating conditions and run at pH 8.9 (Davis, 1964), the other using dissociating conditions and run at pH 3.5 in 9 M urea (Parish and Marchalonis, 1970). Gels from both systems were stained in a solution consisting of 7 % (v/v) acetic acid, 0.25 % (w/v) amido black and 0.5 % (w/v) HgCl₂. Destaining was performed electrophoretically.

2.1.5 Immunoelectrophoresis

This technique was performed by the method of Scheidegger (1955). The gels contained 1.5 % agar in barbital buffer, pH 8.2 ($\mu = 0.05$).

2.1.6 Radioiodination

Soluble proteins were labeled with carrier-free Na [¹²⁵I] (IMS3; The Radiochemical Centre, Amersham) or with Na [¹³¹I] (Code 11B1, Australian Atomic Energy Commission, Lucas Heights, Sydney) by direct oxidation with chloramine-T (Greenwood, Hunter and Glover, 1963; Ada, Nossal and Pye, 1964). To 5-50 μ l of protein solution (0.2-5 mg/ml in DBSS or Tris buffer) was added 1-5 μ l Na [¹²⁵I] or Na [¹³¹I] (< 500 μ Ci) and

chloramine-T to a final concentration of 0.1-0.5 mM. After 5-10 min. at room temperature, $\text{Na}_2\text{S}_2\text{O}_5$ was added in 3 to 4-fold molar excess with respect to chloramine-T, followed by "carrier" NaI to 10 mM. The resulting solution was diluted (usually 10 to 20-fold with DBSS or Tris buffer containing 10 % (v/v) foetal calf serum (FCS), or normal rabbit serum (NRS) and dialyzed at room temperature against buffer containing 15 mM NaN_3 until most unreacted iodine had been removed. Labeling efficiencies were generally 70-95 % and specific activities of 1-50 $\mu\text{Ci}/\mu\text{g}$ protein were used. Iodination had no measurable effect on antibody activity as labeled antibody retained its ability to bind specifically to the antigen against which it was prepared (Fig 2.6). Henceforth, unless stated otherwise, "labeled" refers to ^{125}I -labeled.

Cells were iodinated in either of two ways: (a) Using chloramine-T oxidation as described above, but replacing the protein solution with a cell suspension. After reaction, labeled cells were washed by centrifugation (500 g, 10 min.) and resuspension in isotonic media. (b) Using lactoperoxidase. This enzyme was purified by Mr. K. Clarke from fresh skimmed cows milk by $(\text{NH}_4)_2\text{SO}_4$ precipitation and carboxymethylcellulose chromatography as described by Marchalonis (1969). The purified enzyme ($E_{412}/E_{280} = 0.44$) was stored at -20°C in 0.2 ml aliquots of 0.5 mg/ml. Details for both methods are given in the text.

For direct measurement of radioactivity, samples were counted individually to within 5 % error in a Packard Model 3002 Tri-Carb Scintillation Spectrometer. Counting efficiency for ^{125}I was approximately 60 %.

2.1.7 Autoradiography

This technique was carried out according to Byrt and Ada (1969). Briefly, cell smears were dipped in Kodak NTB-2 photographic emulsion at 42°C , dried vertically and stored in the presence of anhydrous CaSO_4 at 4°C for 1-28 days. After development, the slides were stained in 5 % Giemsa.

2.1.8 Radioimmunoassay

In this technique, the precipitation of labeled antigen (routinely, 1-10 ng) is tested by mixing antigen with twofold serial dilutions of rabbit antiserum. A curve of

precipitation versus antiserum dilution is thus derived. In addition, soluble substances similar or related to the labeled antigen can be tested for their ability to inhibit precipitation of a constant amount of labeled antigen by a given dilution of antiserum. The amount of inhibitor present in a preparation can thus be estimated.

The method is described in detail elsewhere (Parish, Wistar and Ada, 1969). Two diluents were used: BSA-dil, which was 3 % (w/v) bovine serum albumin in 0.05 M Tris-HCl, pH 7.4 containing 15 mM NaN_3 ; and S-dil, which was BSA-dil containing 10 % (v/v) NRS. In some experiments, Nonidet P-40 (NP40) was included in the diluents at 0.5-5 % (v/v) final concentration. At these concentrations, this nonionic detergent dissolves the cell plasma membrane but has no effect on the formation of (Ig/anti-Ig antibody) complexes as measured by this technique. A typical inhibition curve is shown (Fig 2.1) in which 44 ng of sheep IgG or 3800 ng of sheep IgM was needed to inhibit by 50 % the precipitation of ^{125}I -labeled sheep IgG by rabbit anti-sheep- γ -chain serum. Thus the IgM preparation contained 1.2 % (w/w) IgG.

Similarly, inhibitors present on particulate supports can be detected and measured. To estimate the class and amount of Ig chain equivalents exposed on the surface of lymphocytes and able to bind anti-Ig antibody, washed lymphocytes (10^8 cells/ml) in DBSS containing 1 % NRS and 15 mM NaN_3 were diluted serially in twofold steps with the same medium. Portions (50 μl) of each dilution were mixed with 10 μl of diluted antiserum (anti- μ or anti- γ) and incubated overnight at 4°C with gentle agitation. Assays were done in the presence and absence of NP40 (1 % v/v). As standards, dilutions of IgM or IgG were used instead of cells. After incubation, the cells were pelleted and 20 μl of each supernatant was mixed with 50 μl of ^{125}I -labeled IgM or IgG and treated as for the direct precipitation assay (Parish, Wistar and Ada, 1969). Controls included cells plus diluent (for minimal precipitation) and diluent plus antiserum (maximum precipitation).

2.1.9 Gradient centrifugation

This was performed using freshly-prepared linear sucrose density gradients. In velocity sedimentation experiments,

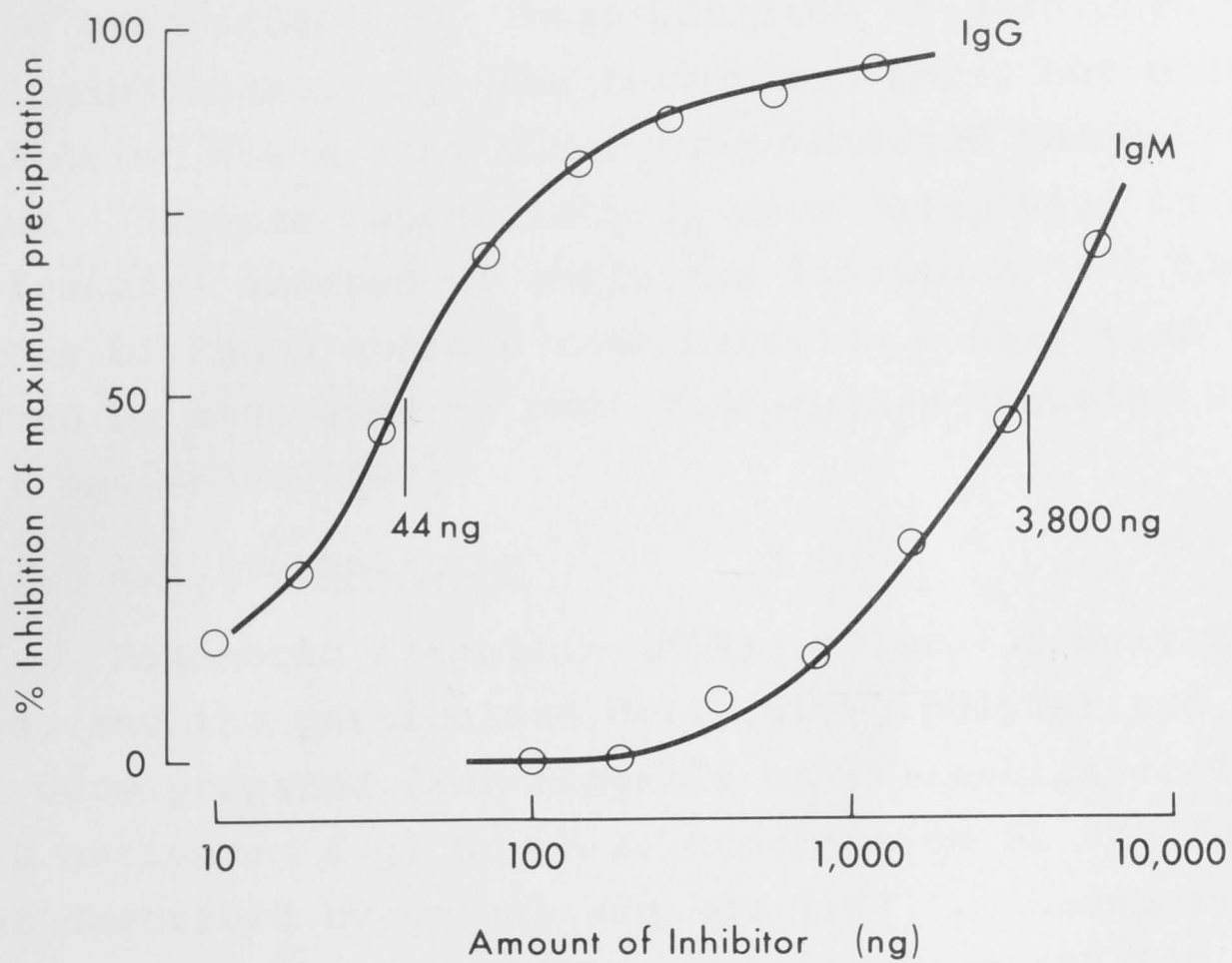


Figure 2.1: Radioimmunoassay curves illustrating the ability of unlabeled sheep IgG or IgM to inhibit the precipitation of ^{125}I -labeled IgG (10 ng) by a fixed amount of anti- γ chain serum.

gradients of 10 % to 30 % (w/v) sucrose were employed. For equilibrium density centrifugation, gradients varying between 20 % and 60 % (w/v) sucrose were used. Details are provided in the text. Samples were layered on to gradients immediately prior to centrifugation. Fractionation of gradients was accomplished usually by tube bottom puncture, but otherwise by collection via a fine glass tube inserted through the gradient. Sucrose concentrations were calculated by comparing the refractive indexes of collected fractions with those of solutions of known sucrose concentration. Densities were estimated by reference to published tables (Dawson, Elliott, Elliott and Jones, 1969).

2.1.10 Antigens

Monomeric *flagellin* (MON), molecular weight 40,000 daltons, and its particulate derivative, polymerized flagellin (POL), were prepared from flagella of *Salmonella adelaide* SW 1338 (H antigen: f,g) or of *S. typhimurium* SL 870 (H antigen: 1,2) as described by Nossal and Ada (1971). *Haemocyanin* (HCY), a pentamer of molecular weight approximately 450,000, was crystallized from the haemolymph of the Southern Australian crayfish, *Jasus lalandii* (Moore, Henderson and Nichol, 1968). It was stored at -20°C as a 100 mg/ml solution in water. *TMVP*, the unit protein of tobacco mosaic virus, strain *vulgare*, was obtained through the courtesy of Dr. E. Benjamini.

2.1.11 Animals

Sheep were randomly bred merino virgin ewes or wethers. Inbred CBA/H mice and outbred chickens and rabbits were obtained from the John Curtin School of Medical Research animal colony.

2.1.12 Cell Suspensions

2.1.12.1 Red Cells

Sheep red blood cells (SRBC) were prepared from blood drawn by venipuncture. Red cells were washed at least 4 times in saline and the buffy coat removed after each centrifugation. Chicken red cells (CRBC) were prepared as for SRBC.

2.1.12.2 Lymphoid cells

Sheep lymphocytes were obtained from lymph (collected

continuously in plastic bottles containing 200 i.u. heparin and 2000 i.u. penicillin in 2 ml saline) by cannulation of the efferent duct of popliteal, prescapular or lumbar nodes of sheep (Hall and Morris, 1962). Routinely, cells in lymph collected for periods of up to 24 h were washed once in saline and thrice at 4°C in DBSS containing 10 % (v/v) FCS and 15 mM NaN_3 . After washing, cell viability as measured by trypan blue exclusion was 95-100 %. Cell populations from unstimulated sheep consisted of lymphocytes (95-100 %), polymorphonuclear neutrophils, eosinophils, large basophilic and plasma cells (together < 5 %), macrophages (< 1 %) and erythrocytes (< 1 %) (Smith, McIntosh and Morris, 1970).

Mouse spleen or lymph node cell suspensions were prepared by cutting mouse spleens or mesenteric lymph nodes into small pieces and gently teasing the pieces through a stainless steel sieve (80 mesh x 0.005 in. diameter wire) into cold DBSS containing 10 % FCS. The cells were washed thrice in this medium, when cell viability was normally 90-95 %. Cells were counted on a haemocytometer (AO Instrument Co., Buffalo, N.Y.).

2.1.12.3 Lymphocyte cultures

For short-term cultures, lymphocytes were suspended at 1×10^7 cells/ml in Eagle's medium supplemented with sterile NaHCO_3 (40 mM), FCS (5 %), and penicillin and streptomycin (each 100 i.u./ml). The suspensions were gassed with 10 % CO_2 /7 % O_2 /87 % N_2 (Commonwealth Industrial Gasses, Sydney) and incubated at 0°C or 37°C. Cell viability as measured by trypan blue exclusion was normally > 80 % after 20 h incubation.

2.1.12.4 Conditions for labeling lymphocytes with radiolabeled proteins

Washed lymphocytes were suspended (normally at 10^8 cells/ml) in DBSS containing 10 % FCS (if labeling with antigen) or 10 % NRS (if labeling with rabbit antibody) and 15 mM NaN_3 . All subsequent operations were performed at 0-4°C. Labeled antigen or antibody was added to the required concentration (50-500 ng/ml) and the cells incubated, usually for 60 min., before being washed. For quantitative measurement of labeled protein bound to the cells, triplicate samples were taken

before and after cell washing and counted for bulk radioactivity. It has been shown elsewhere that using this procedure, labeled antigen (Mandel, Byrt and Ada, 1969) or antibody (Unanue, Perkins and Karnovsky, 1972a,b) attached to the cell remains almost exclusively at the cell surface. Labeled antibodies used for sedimentation experiments were first sedimented, after iodination, through density gradients and the purified 7S antibodies then used to label cells.

2.1.12.5 Cell disruption techniques

A variety of methods including hypotonic lysis, mechanical or ultrasonic disruption, or detergent treatment were employed to disintegrate red blood cells or lymphocytes. Sonic rupture was achieved with an MSE/Mullard probe-type ultrasonicator unit possessing a frequency of 20,000 Hz. For mechanical disruption, cells were swollen by suspension in hypotonic solution, placed in an ice-cooled glass Dounce homogenizer tube and ruptured by at least 10 complete strokes with a tight-fitting glass rod. All details of disruption procedures, including the composition of the various solutions used, are described in the text.

In some experiments (Chapter 6), cell extracts were prepared by dissolving cells labeled with antibody in a solution containing NP40. Solution (*i.e.* label not sedimented by 1000xg, 15 min.) from cells was effected by NP40 concentrations of ≥ 0.05 % (v/v). The extent of solubilization of labeled anti-Ig antibody associated with membrane (Ig) components depended on disruption conditions, with a maximum 60-80 % solubilized within 30 min. at 0°C in 0.05 % NP40/5 mM EDTA/5 mM Tris-HCl, pH 7.5. The remaining 20-30 % of label remained closely associated with insoluble cellular debris and neither higher detergent concentrations (≤ 5 %) nor longer extraction times were effective in further solubilization. Most satisfactory solution was achieved at low ionic strengths in the presence of EDTA, and a solution containing 0.1 % NP40/5 mM EDTA/5 mM Tris-HCl, pH 7.5 was routinely used. This treatment caused no discernable breakdown of carrier 19S IgM to its 7S subunits.

2.1.13 Antibody titrations

2.1.13.1 Anti-SRBC or anti-CRBC (Haemagglutination)

Doubling dilutions of serum (anti-SRBC or anti-CRBC) were titrated against a final concentration of 0.5 % (v/v) washed SRBC or CRBC, respectively, in V-cupped perspex trays. End-points were taken as 50 % agglutination after 4-6 h incubation at room temperature. For inhibition experiments, anti-SRBC or anti-CRBC serum was diluted in serial twofold steps. To 50 μ l of each serum dilution was added (firstly) 25 μ l of the sample to be tested and (secondly) 25 μ l of 0.5 % (v/v) SRBC or CRBC. End-points were again taken as 50 % agglutination.

2.1.13.2 Anti-sheep-lymphocyte globulin (ALG)

ALG was titrated as for anti-SRBC serum, but using sheep lymphocytes instead of SRBC. Agglutination was visualized under low power magnification. Inhibition experiments were performed as for anti-SRBC inhibition.

2.1.13.3 Anti-POL or anti-HCY (Passive haemagglutination of POL- or HCY-coated SRBC)

A modification of the method of Gold and Fudenberg (1967) was employed. A 0.1 % (w/v) solution of $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ in saline was prepared and adjusted to pH 5.0 with 1 N NaOH daily for one week. This solution was diluted with saline to give a 0.01 % solution which was suitable for use in the coating procedure for up to two weeks.

The standard protocol involved the addition of 50 μ l POL or HCY to 2.5 ml 10 % washed SRBC followed by 0.6 ml of 0.01 % CrCl_3 solution and rapid mixing. After standing at room temperature for 5 min, the cells were washed twice in 10 ml saline and a 2 % suspension of the final cell pellet made in saline. Aliquots (50 μ l) of this suspension were added to twofold dilutions of serum (50 μ l aliquots in saline containing 5 % FCS) in V-cupped perspex trays. The serum/SRBC mixtures were allowed to settle for 4-6 h at room temperature before 50 % agglutination was taken as the end-point of each titration.

2.2 Immunoglobulins, antisera and antibodies

2.2.1 Sheep IgG and serum macroglobulin

Sera from 5 sheep (one of which was immunized 4 weeks before bleeding with 1 mg POL 1338 in 0.5 ml complete Freund's adjuvant (Difco), intradermally) were pooled and centrifuged (10,000xg, 30 min.) to remove lipoproteins. Aliquots were then applied to a column of Sephadex G-200 (Pharmacia, Sweden) and eluted with DBSS containing 15 mM NaN_3 . The first (excluded) and second peaks from several chromatographs were respectively pooled, concentrated by pressure dialysis and re-applied separately to the column. Protein (A_{280}) and anti-1338 antibody (passive haemagglutination) were measured. The elution profiles are shown in Fig 2.2. Purified macroglobulin (excluded material, Fig 2.2b) containing anti-1338 IgM antibody was concentrated and stored at -20°C .

IgG was further purified from fractions containing anti-1338 antibody (Fig 2.2c) by dialysis against 20 mM sodium phosphate, pH 8.0 and passage through a column of DEAE-cellulose equilibrated and eluted with the same buffer. The effluent, containing a single protein peak, was concentrated and subsequently shown to consist of pure IgG (see below), as has been found by others (Harrison and Mage, 1967; Jonas, 1969). In subsequent IgG preparations, serum was made 40 % saturated with neutralized $(\text{NH}_4)_2\text{SO}_4$ and the resulting precipitate was dissolved, dialyzed against 20 mM phosphate, pH 8.0 and chromatographed as above on DEAE-cellulose. No attempt was made to fractionate the IgG into its γ_1 and γ_2 subclasses (Harrison and Mage, 1967; Heimer, Clark and Maurer, 1969). Analytical centrifugation in a Spinco Model E ultracentrifuge revealed single components in the IgG and macroglobulin preparations, sedimenting with an $S_{20,w}$ (uncorrected for protein concentration) of 7.0 (IgG) and 16.9 (macroglobulin).

2.2.2 Reduction of sheep IgG: Separation of γ and L chains

Sheep IgG (18 mg/ml) was reduced under N_2 with 0.75 M 2ME for 90 min. at room temperature (Williams and Chase, 1967). After cooling in ice, iodoacetamide (1 vol, 0.8 M) was added and the pH kept > 8 by the dropwise addition of triethylamine.

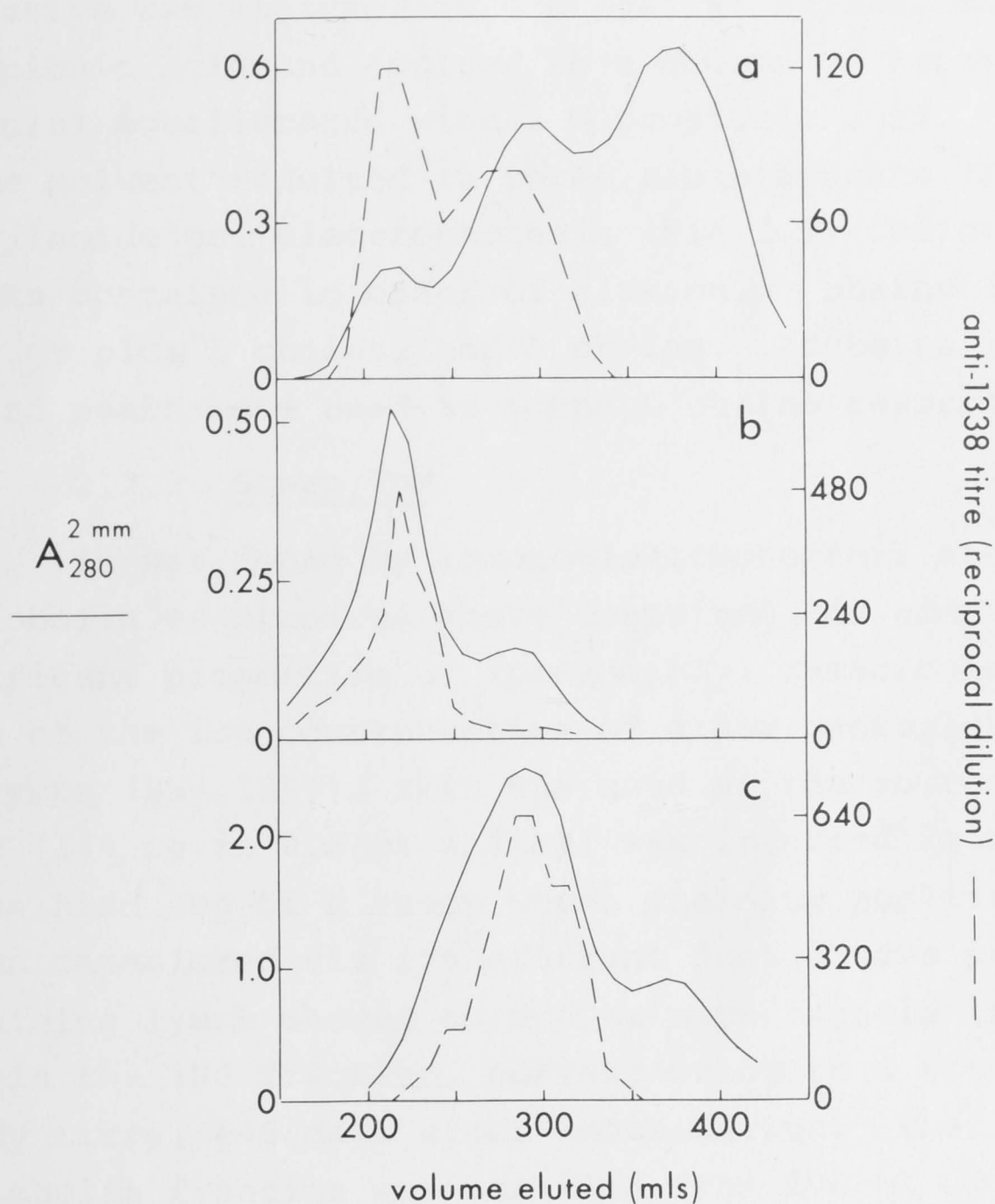


Figure 2.2: Elution profiles of (a) sheep serum; (b) excluded material (first peak) from (a); and (c) material in the second peak from (a), after chromatography through Sephadex G-200. The column (71x3.3 cm) had a bed volume of 635 ml and an excluded volume of 215 ml. Combined fractions from (a) were concentrated by pressure dialysis before being separately reappplied to the column in (b) and (c). The buffer was DBSS containing 15 mM NaN_3 . Protein was measured by optical density (A_{280}) and anti-1338 antibody by passive haemagglutination.

The solution was dialyzed at 4°C against saline, then against 1 N propionic acid and applied to a column of Sephadex G-100 (Pharmacia) equilibrated with 1 N propionic acid. Elution with the same solvent resulted in three protein peaks (Fig 2.3). Polyacrylamide gel electrophoresis (Fig 2.3) indicated that the peaks contained in order of elution, γ chains (presumably dimers), γ plus L chains, and L chains. Proteins in the first and third peaks were used as γ and L chains respectively.

2.2.3 Sheep IgM

It was found by immunoelectrophoresis that serum macroglobulin as prepared above contained, in addition to IgM, a significant proportion of (presumably) α -macroglobulin. Because of the low concentration of other macroglobulins in sheep lymph (Hay, 1970), this was used as the source of IgM. POL 870 (144 μ g in 0.5 ml saline) was injected intradermally into the hind leg of a sheep whose draining popliteal node had been cannulated via its efferent duct 3 days previously. The draining lymph showed an approximate twofold increase (A_{280}) in the 19S fraction, corresponding to a rise in 19S antibody titre, 4-5 days after immunization. The 19S macroglobulin fraction was purified from 100 ml of lymph (collected on the 5th day post-immunization) by repeated sedimentation centrifugation until it was free of more-slowly sedimenting material. The separation obtained by this technique is illustrated in Fig 2.4.

On immunoelectrophoresis, antisera prepared against this IgM concentrate (19S macroglobulin) produced two distinct precipitin lines, the major one against α -macroglobulin and the minor against IgM. To separate IgM from 19S α -macroglobulin, advantage was made of the dissociation of IgM into 7-8S subunits at low concentrations of 2ME (Mukkur and Inman, 1970; Suzuki and Deutsch, 1967). After reduction (0.05 M 2ME, 0.1 M Tris-HCl, pH 8.2 for 30 min. at room temp.), the 19S fraction was resedimented through a sucrose gradient containing 0.05 M 2ME in 0.1 M Tris-HCl, pH 8.2. The result is shown in Fig 2.5. It can be seen from the immunoelectrophoresis patterns (Fig 2.5) that material sedimenting at 7S in the presence of 2ME reacted with anti-L-chain serum but was free of contaminating α -macroglobulin. The latter continued to sediment in 2ME at 17S

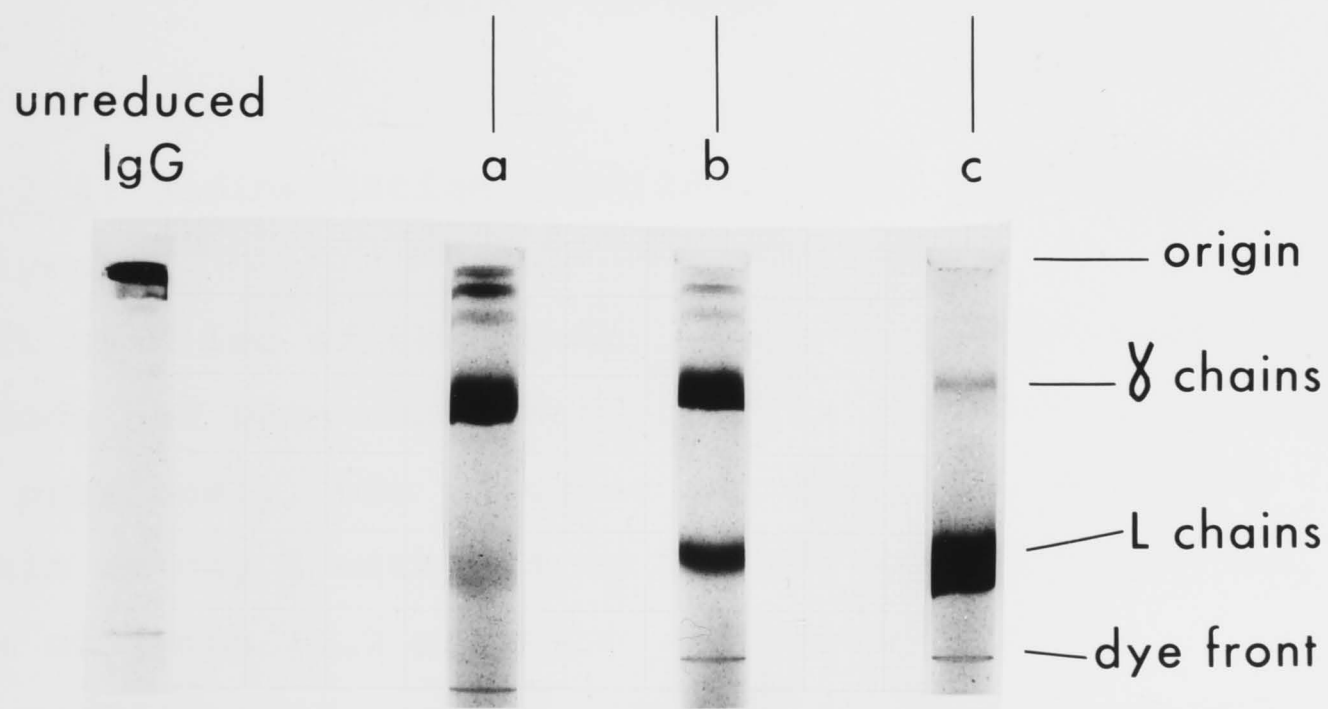
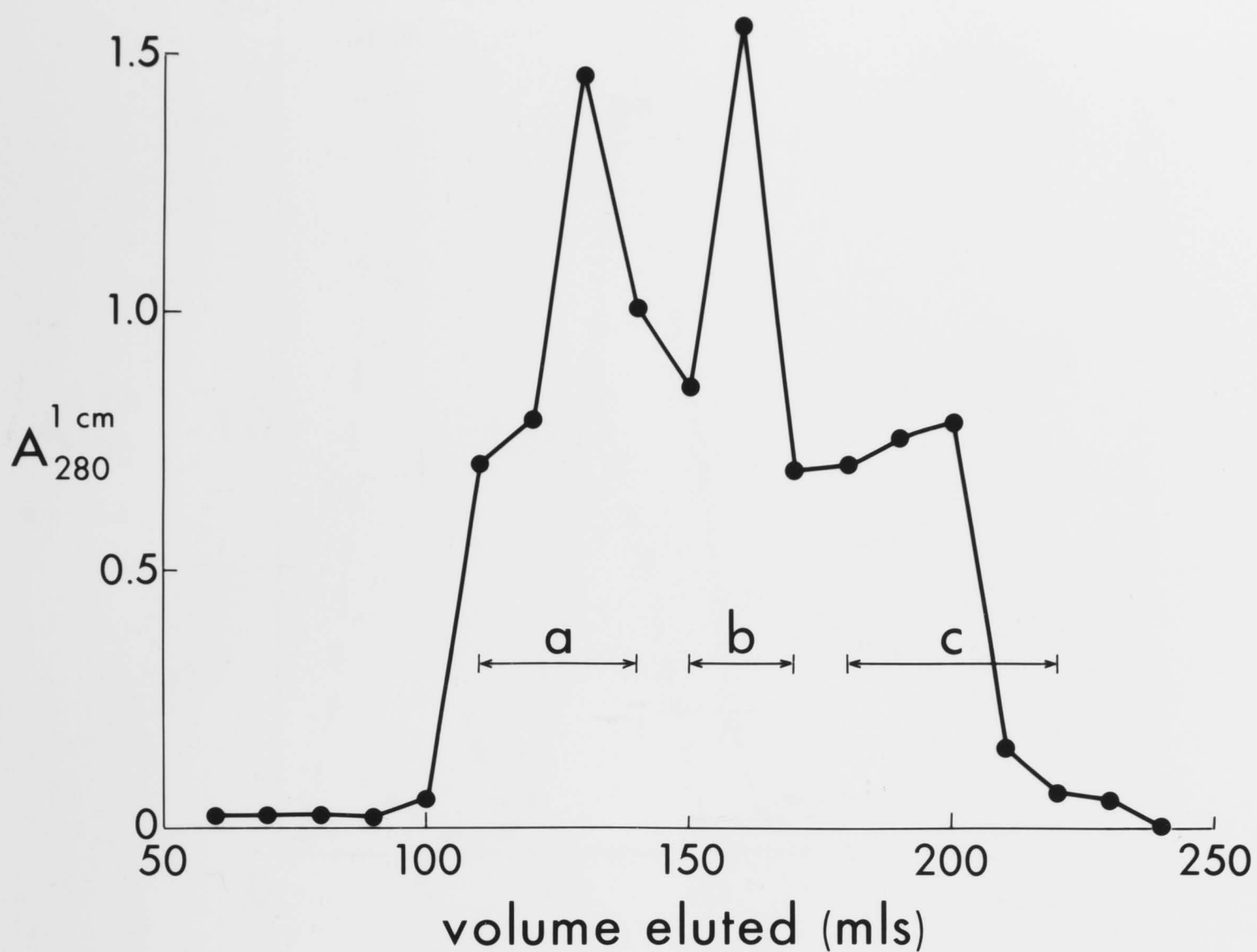


Figure 2.3: Top: Elution profile of reduced, acetylated sheep IgG after Sephadex G-100 chromatography in 1 N propionic acid. The column (50 X 1.86 cm) had a 135 ml bed volume. Fractions were combined as indicated (a,b,c), dialyzed at 4°C against several changes of DBSS and concentrated by pressure dialysis.

Bottom: Analysis of unreduced sheep IgG, and fractions a, b and c from above, by polyacrylamide gel electrophoresis in 9 M urea, pH 3.5.

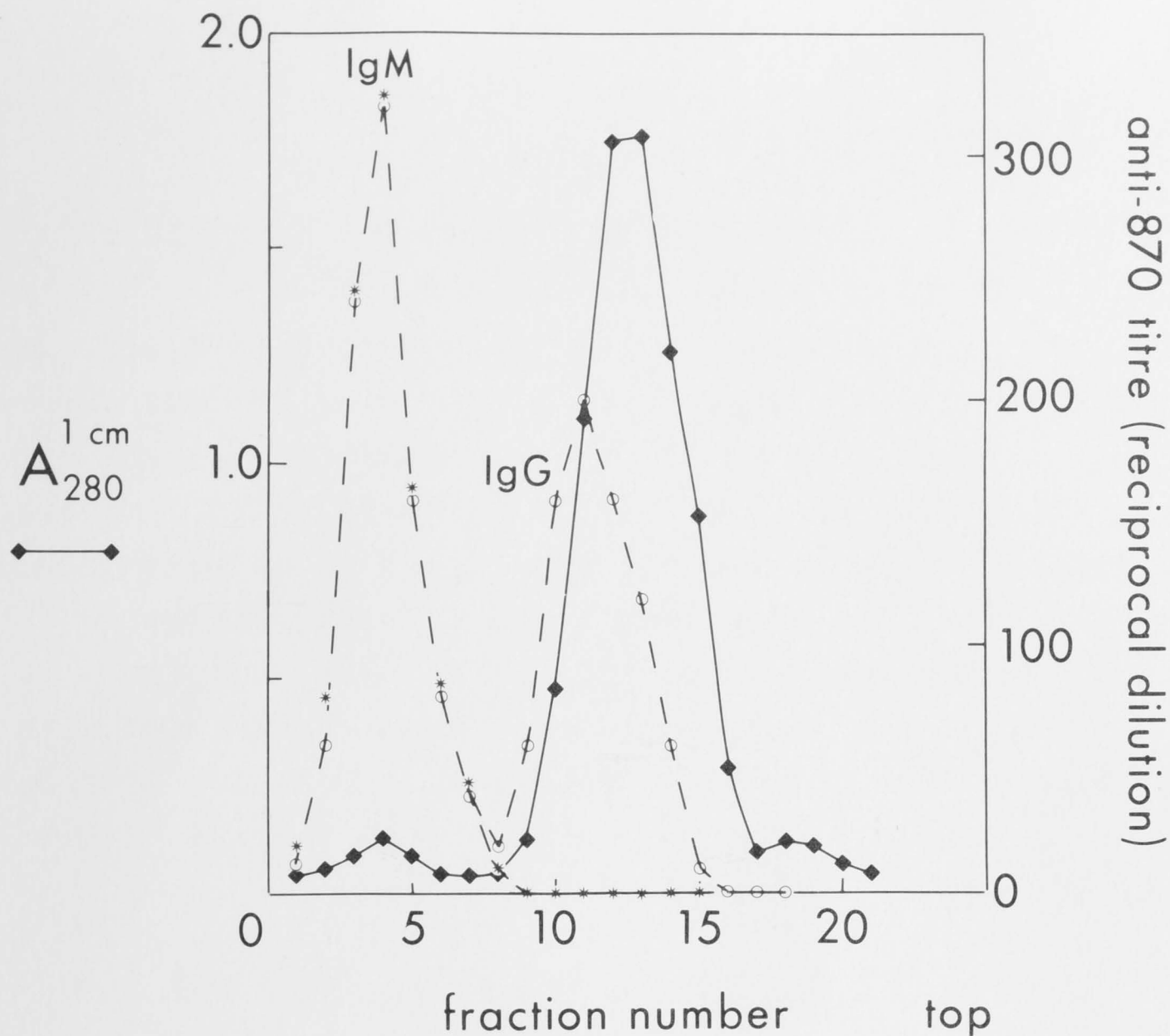


Figure 2.4: Sedimentation profiles of POL 870-immune sheep lymph.

The left hind leg of the sheep, whose left popliteal lymph node had been cannulated via its efferent duct 3 days previously, was injected intradermally on day 0 and again on day 6 with 144 mg POL 870 in 0.5 ml saline. Samples of lymph (0.2 ml) were sedimented through linear gradients of sucrose (10 - 30 %, w/v) dissolved in DBSS (SW50.1 rotor; 45,000 rpm, 9 h, 5°C). Anti-870 antibody titrations were done by passive haemagglutination:

* -- * -- * lymph collected on day 5
 O -- O -- O " " " " 9

IgM and IgG antibody peaks are indicated. The A_{280} profile was similar for each lymph sample.

Figure 2.5

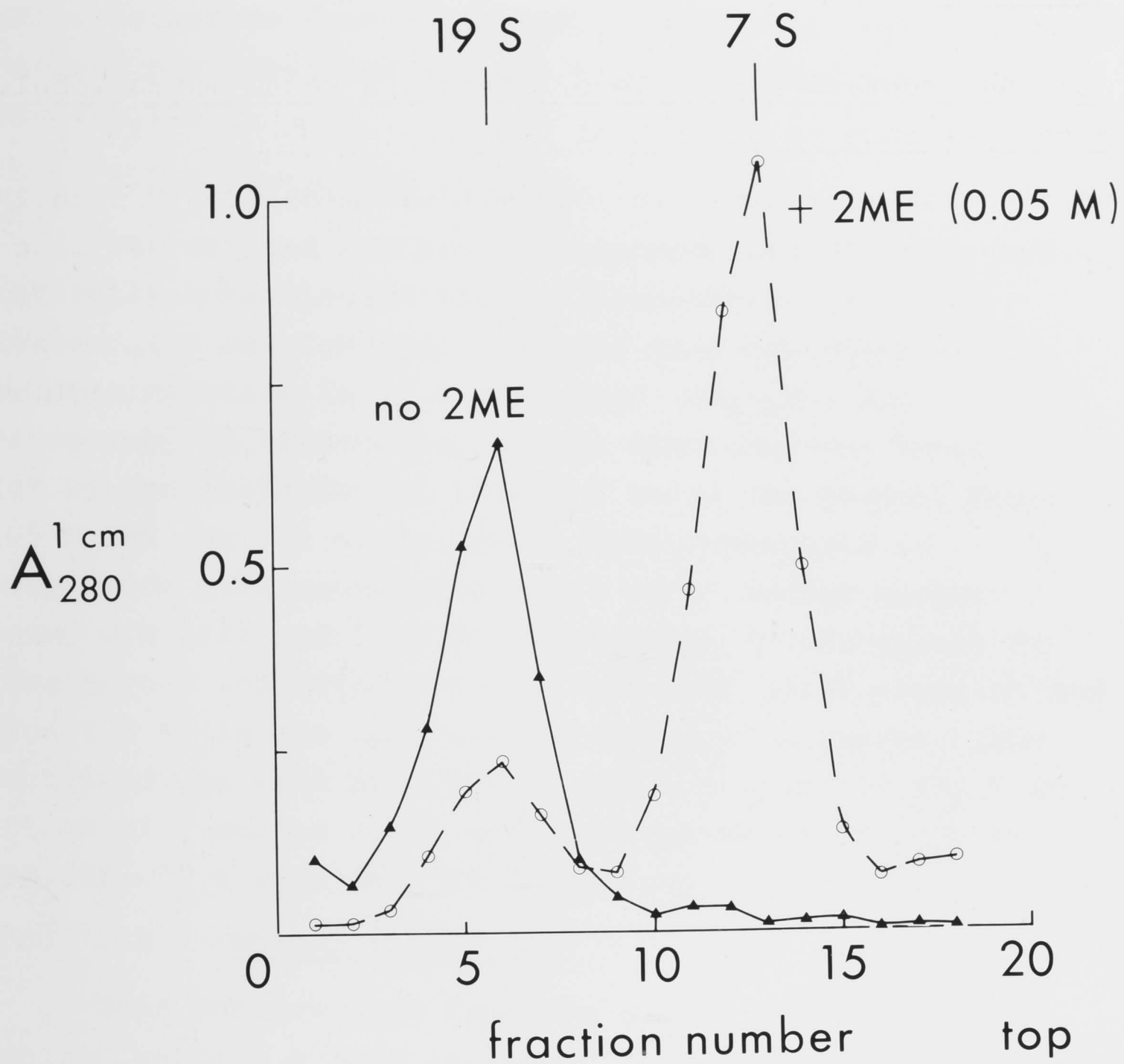
Top: Sedimentation profile of sheep 19S serum macroglobulin in the presence or absence of 2-mercaptoethanol

Purified 19S macroglobulin (0.3 ml) was made 0.05 M with 2ME and incubated at room temperature for 15 min. It was then sedimented through a 10 % - 30 % (w/v) sucrose gradient containing 0.15 M Tris-HCl (pH 8.2), 0.05 M 2ME (SW50.1 rotor; 48,000 rpm, 10°C, 7 h). As a control, 0.1 ml of macroglobulin was mixed with 0.2 ml of Tris-HCl, pH 8.2 and sedimented through a similar gradient containing no 2ME. Fractions (0.3 ml) were collected by tube puncture. Each was diluted with saline, 0.6 ml (2ME fractions) or 0.3 ml (control fractions), and the A_{280} measured. The position of 19S IgM and 7S IgG antibody markers is indicated.

Bottom: Immuno-electrophoretic analysis of fractions from sedimentation (above)

Fractions 4-7 and 10-15 (inclusive) from each gradient were pooled and dialyzed against 0.15 M Tris-HCl, pH 8.2. A sample of each preparation was then electrophoresed (90 min., 200 V, 5 mA per gel) and subjected to immunodiffusion against the indicated antisera. The gels were washed and stained as described in Fig 2.7. The direction of electrophoresis is indicated (anode, +).

* Sample #6 = fractions 4-7 (inclusive)
" #13 = " 10-15(")



<u>antiserum</u>		<u>antiserum</u>		
<u>sample</u> *				<u>sample</u> *
	anti-(sheep serum)		anti-(19 S)macroglobulin	
#6 (+2ME)		+		#6 (no 2ME)
	anti-sheep L chain		anti-(sheep serum)	
#13 (+2ME)		+		#13 (+2ME)
	anti-(sheep serum)		anti-(19 S)macroglobulin	

and showed no reaction with anti-L-chain serum. Purified IgM hereafter refers to reduced 7S IgM, although it should be noted that it was not alkylated and may have repolymerized (Suzuki and Deutsch, 1967).

2.2.4 Mouse IgG and IgM

The skilled technical assistance of Mrs. B.J. Howlett is gratefully acknowledged for the preparation of these materials. IgG and IgM were prepared from CBA mouse serum, IgG by precipitation in 40 % saturated $(\text{NH}_4)_2\text{SO}_4$ and chromatography on DEAE-cellulose (as described for sheep IgG) and IgM by sedimentation of purified serum 19S macroglobulin in 0.05 M 2ME (as for sheep IgM). Electrophoresis in polyacrylamide gels containing 0.1 % (w/v) sodium dodecyl sulphate, 2 M urea and 2 mM dithiothreitol (performed by Dr. A.T. Bradburne) indicated γ chains (53 % of total protein) and L chains (31 %) in the IgG, with no trace of μ chains. The IgM contained, as well as μ (34 %) and L chains (23 %), a small proportion of γ chains (3 %) and other proteins (11 % with MW > 90,000; 29 % with MW < 20,000 daltons).

2.2.5 Normal rabbit IgG

This was prepared from the pooled sera of several unimmunized rabbits by precipitation in 40 % saturated $(\text{NH}_4)_2\text{SO}_4$ and chromatography on DEAE-cellulose, as described for the preparation of sheep IgG.

2.2.6 Rabbit antisera and antibodies

2.2.6.1 Anti-sheep-Ig sera

Rabbits were injected intradermally with 1 mg Ig protein emulsified in complete Freund's adjuvant (Difco) followed 3 weeks later with 1 mg in incomplete Freund's adjuvant (Difco). The animals were bled from the ear 3-5 weeks after the second injection and all sera were inactivated at 62°C for 15 min. Antisera were prepared against sheep IgM, IgG, γ chains, L chains, 19S macroglobulin and whole serum.

To obtain antisera specific for individual heavy chains (μ or γ), appropriate antisera were chromatographed on columns of Sepharose 4B (Pharmacia) to which IgM or IgG had been covalently attached (Cuatrecasas, Wilchek and Anfinsen, 1968;

Boegman and Crumpton, 1970). Thus, specific anti- μ -chain serum was obtained by passing anti-IgM serum through a column of Sepharose conjugated with IgG (Seph(IgG)) and the effluent collected. Similarly, specific anti- γ -chain serum was prepared by passing anti-IgG or anti- γ -chain serum through a column of Sepharose conjugated with IgM (Seph(IgM)).

2.2.6.2 Specific anti-sheep-Ig-chain antibodies

Rabbit IgG antibodies with specificities directed against sheep μ , γ or L chains were prepared from their respective antisera. Thus, anti- μ -chain antibody was absorbed on to a column of Seph(IgM) followed by elution with 1 N propionic acid. Anti- γ -chain antibody was similarly absorbed on to Seph(IgG). Anti-L-chain antibody was absorbed and eluted, first on Seph(IgG) and then on Seph(IgM). The specificity of this type of absorption is illustrated in Fig 2.6, where radioiodinated IgG antibodies specific against sheep L or γ chains, mouse L chains or sheep lymphocytes were individually absorbed to and eluted from a column of Seph(sheep IgG). Only antibodies against sheep L or γ chains were significantly absorbed to the column.

2.2.6.3 Anti-mouse-Ig sera

Rabbit anti-mouse-IgG and anti-IgM sera were prepared by a regime identical to that for anti-sheep-Ig sera. Sera were made specific for mouse μ or γ chains by passage through columns of Seph(mouse IgG) or Seph(mouse IgM), similar to the preparation of specific anti-sheep-Ig-chain sera. When tested by radioimmunoassay, anti-mouse- μ -chain serum precipitated 50 % of ^{125}I -labeled IgM (10 ng) at a 1/700 dilution, whilst precipitating < 5 % of labeled IgG (10 ng) at a dilution of 1/2. Anti- μ -chain serum was therefore quite specific for mouse μ chains. Anti-mouse- γ -chain serum precipitated 50 % of labeled IgG (10 ng) at 1/1600 dilution. However it also precipitated 50 % of labeled IgM (10 ng) at 1/2 dilution, and 10 % at 1/10. Thus anti- γ -chain serum contained trace activity against IgM, probably as anti-L-chain.

2.2.6.4 Specific anti-mouse-L-chain antibody

This reagent was kindly prepared by Mrs. B.J. Howlett

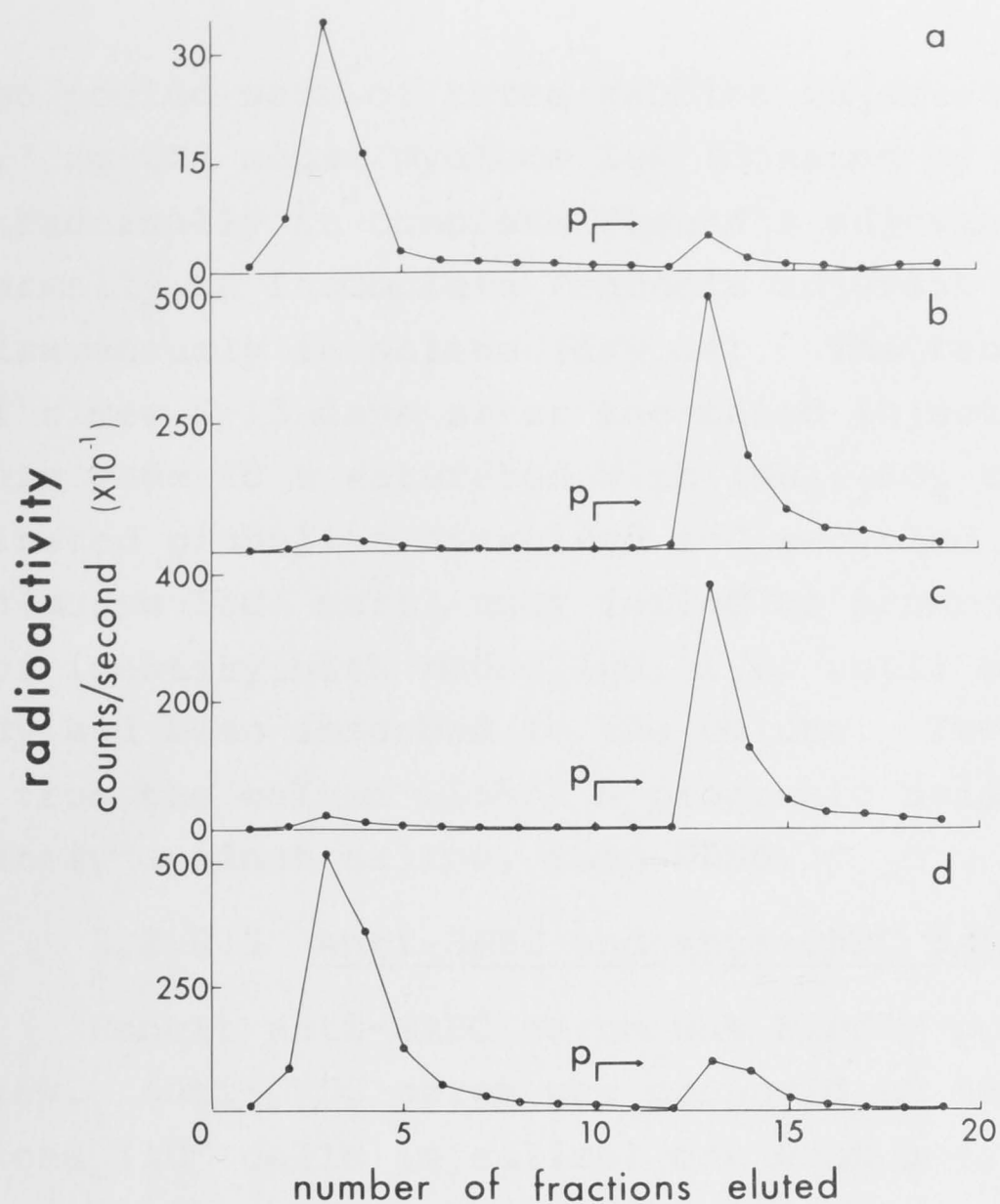


Figure 2.6: Affinity chromatography of ^{125}I -labeled rabbit antibodies on a column of Sepharose 4B to which sheep IgG had been covalently attached.

Before applying any sample, the column was washed with 1 N propionic acid until the effluent had zero A_{280} and contained no radioactivity, and then with DBSS until the effluent was at neutral pH. After a sample was applied, the column was washed at 4°C with aliquots of DBSS (resulting in 10 fractions) and then (from p) with 1 N propionic acid to elute bound antibodies.

The ^{125}I -labeled antibodies applied and their percentage recovery (radioactivity) were:

	<u>% Recovery</u>
a) anti-mouse-L chain	103
b) anti-sheep-L chain	55
c) anti-sheep- γ chain	63
d) anti-sheep-lymphocyte globulin (ALG)	80

from the pooled sera of three rabbits injected on 3 occasions with 0.3 mg CBA mouse myeloma IgA (donated by Dr. N.L. Warner): (1) intradermally in complete Freund's adjuvant (day 0), (2) intradermally in incomplete Freund's adjuvant (day 31) and (3) intravenously in saline (day 54). The rabbits were bled several times 6-13 days after the third injection. The pooled sera were made 40 % saturated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitated globulins dissolved and recycled through a column of Seph(mouse IgG) until they failed to produce immunodiffusion lines of identity with mouse IgG, *i.e.* until most anti-L-chain antibody had been absorbed to the column. The antibody was eluted from the column with 1 N propionic acid and dialyzed immediately against saline, then DBSS.

2.2.6.5 Anti-SRBC and anti-CRBC sera

Rabbit anti-SRBC serum was kindly provided by Dr. F.Y. Liew. Anti-CRBC serum was prepared by two intravenous injections (10^9 cells in saline) one week apart. The animals were bled 4 weeks after the second injection.

2.2.6.6 Anti-sheep-lymphocyte globulin (ALG)

Rabbit anti-sheep-lymphocyte sera were kindly donated by Dr. R. Scollay. They were prepared as follows: each of 3 rabbits was injected with a suspension of lymph cells (10^8 cells in complete Freund's adjuvant, intradermally) collected from the efferent duct of the popliteal nodes of several sheep. After 15 days, the rabbits were injected with 10^8 cells (in DBSS) intravenously and thereafter at 10 day intervals for 8 weeks. Three months after the last injection, they were given a final dose (10^8 cells, intravenously) before being bled by cardiac puncture one week later. Anti-lymphocyte globulin (ALG) was purified from the pooled sera by precipitation in 40 % saturated $(\text{NH}_4)_2\text{SO}_4$, chromatography on DEAE-cellulose and passage through columns of Seph(sheep IgG) and Seph(IgM) to remove anti-Ig antibodies (Fig 2.6). ^{125}I -labeled ALG bound specifically to 100 % of lymphocytes from efferent lymph, as determined by autoradiography.

2.2.7 Purity of sheep Igs and specificity of rabbit anti-sheep-Ig sera

As an initial test for cross-reactivity, sheep serum

and purified IgM, IgG, L chains and γ chains were immuno-electrophoresed against anti-L, anti- γ and anti- μ -chain sera. The gels are shown in Fig 2.7 and the results summarized in Table 2.1. Anti- μ -chain serum, though it reacted against α -macroglobulin and IgM, did not react against L or γ chains. It should be noted that the amount of α -macroglobulin in the IgM preparation was insufficient to produce a line of precipitation against anti- μ -chain serum. Anti- γ -chain serum also appeared specific, showing no activity against IgM or against L chains. The anti-L-chain serum, however, showed some activity against γ chains.

By radioimmunoassay, 50 % of ^{125}I -labeled IgM (10 ng) could be precipitated by a 1/900 dilution of anti- μ -chain serum. The same serum, undiluted, precipitated < 2 % of labeled IgG (10 ng). The reverse was true for anti- γ -chain serum, whilst anti-L-chain serum precipitated 50 % of both IgM or IgG at dilutions \leq 1/400. Anti- μ and anti- γ -chain sera were therefore considered specific for μ and γ chains, respectively. Anti-L-chain serum had no detectable anti- μ -chain activity and only trace activity against γ chains.

To determine the extent of cross-contamination of the purified Igs and their component chains, the ability of each to inhibit the precipitation of labeled Ig by specific antisera was determined, with the results shown in Table 2.2. IgM was found to contain 1.2 % IgG (w/w) and 18 % L chain (theoretically, 25 %). IgG contained < 0.08 % IgM and 32 % L chain (theoretically, 31 %). Light chain contained 3.4 % IgG (equivalent to 2.4 % γ chain) and < 0.3 % IgM, whereas γ chain contained 6.1 % L chain and < 0.3 % IgM (μ). The unexpected large amount of γ chain (75 ng) needed to inhibit the precipitation of labeled IgG by anti- γ -chain serum may have been due to polymerization.

2.2.8 Rabbit anti-sheep-Ig Fab fragments:

Preparation and specificity

Pure rabbit IgG antibody (5-10 μg) specific against sheep L, γ or μ chains was labeled with iodine-125, diluted with 0.3-0.5 ml neat NRS (which had been dialyzed against 2 mM EDTA/0.1 M phosphate buffer, pH 7.5) and digested with papain (2-4 h, 37°C) according to Porter (1959). The reaction was

Figure 2.7: Agar gel immunoelectrophoresis of sheep serum and purified sheep immunoglobulins

Samples (0.5 mg protein/ml, except serum which was diluted 1/4) were dialyzed for 3 h against veronal buffer, pH 8.2 prior to electrophoresis (90 min., 240 V, 3.5mA per gel). Undiluted serum (as indicated) was then applied to wells cut parallel to the direction of electrophoresis. After standing at room temperature for 60 h, each gel was washed in saline containing 15 mM azide (5 times, once every 24 h), water (once) and then air-dried. They were then stained with amido black (0.25 % w/v) in 7 % (v/v) acetic acid. The anode (+) indicates the direction of electrophoresis. The results are summarized in Table 2.1.

antiserum

sample*

anti-L chain



IgG

anti- γ chain



IgM

+

anti-L chain

anti-(sheep serum)



IgM

+

anti- μ chain



sheep serum

+

anti-(sheep serum)

anti-L chain



L chains

+

anti- μ chain

anti-(sheep serum)



sheep serum

+

anti-(19 S)macroglobulin



IgM

+

anti-(19 S)macroglobulin

Table 2.1.

Immunoelectrophoresis of sheep immunoglobulins^{a)}

<u>Antigen</u>	<u>Antiserum specificity</u>		
	<u>Anti-L</u>	<u>Anti-γ</u>	<u>Anti-μ</u>
serum	2	1	2
IgM	1	0	1
IgG	2	1	0
L chain	1	0	0
γ chain	1 (v. faint)	1	0

a) The number of precipitation lines visible after immunodiffusion between antisera and the electrophoresed antigens. Antigens were used at 0.5 mg/ml, except γ chain (0.2 mg/ml) and serum (diluted 1/4). Antisera were undiluted.

Table 2.2

Ability of sheep IgG, IgM, L chains and γ chains to inhibit the precipitation of sheep Ig antigens as tested by radioimmunoassay^{a)}

<u>Inhibitor</u>	<u>IgG/Anti-γ</u>		<u>IgM/Anti-μ</u>		<u>L chain/Anti-L</u>	
	<u>ng</u>	<u>% IgG</u>	<u>ng</u>	<u>% IgM</u>	<u>ng</u>	<u>% L chain</u>
IgG	44	(100)	>18000	(<0.08)	530	(32)
IgM	38000	(1.2)	15	(100)	950	(18)
L chain	1300	(3.4)	>4500	(<0.3)	170	(100)
γ chain	75 ^{b)}	-	>5300	(<0.3)	2800	(6.1)

^{a)} The amount of inhibitor (ng) needed to inhibit by 50 % the precipitation of 10 ng of ¹²⁵I-labeled sheep IgG, IgM or L chains by fixed amounts of the indicated antisera. The figures in parentheses are the calculated percentage contamination of each inhibitor by the Ig whose precipitation is inhibited (arbitrarily 100 % for the homologous inhibitor).

^{b)} This value is about 2.3 times greater than expected, possibly because of γ chain dimerization.

stopped by adding solid iodoacetamide and the entire mixture was then sedimented through a density gradient (solvent, DBSS). Fractions containing the 3.5S peak were pooled. This contained labeled Fab (specific for L, γ or μ chains) and labeled Fc piece in an excess of unlabeled Fc and unlabeled (nonspecific) Fab. Separation from Fc was not attempted as monomeric antibody, rather than pure Fab, was required.

The specificity of these reagents is illustrated in Fig 2.8, where labeled Fab, after incubation with sheep serum, was sedimented through a density gradient. Labeled anti- γ or anti- μ -chain Fab showed no labeling of IgM or IgG respectively and anti-L-chain Fab labeled both Igs in the ratio of their respective serum concentrations. This indicated that each reagent was monospecific for its respective Ig chain.

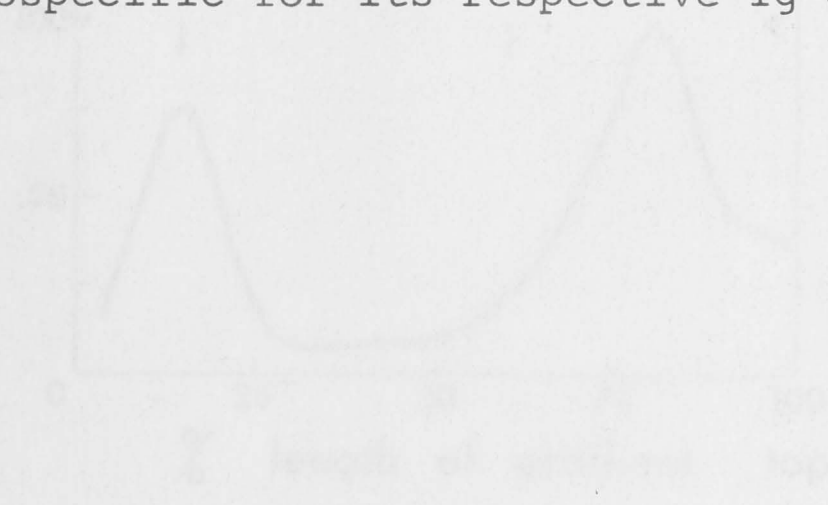


Figure 2.8. Sedimentation patterns of ^{125}I -labeled Fab (10 μl) specific for (a) γ chain, (b) μ chain, and (c) L chain, after incubation with 100 μl of sheep serum. Each sample was sedimented through gradients of sucrose (10 - 30% w/v) dissolved in DBSS containing 0.01% $\text{Na}_2\text{S}_2\text{O}_4$ and 25 mM NaCl . The broken line in (a) indicates the sedimentation position of unlabeled Fab. The positions of 7S IgG and 19S IgM are shown. Labeled material sedimenting at 1.5S after sedimentation of serum is labeled Fc piece and some labeled Fab. The sedimentation patterns are plotted as counts versus time (min) for (a) 1252 cps (b) 1252 cps and (c) 1252 cps.

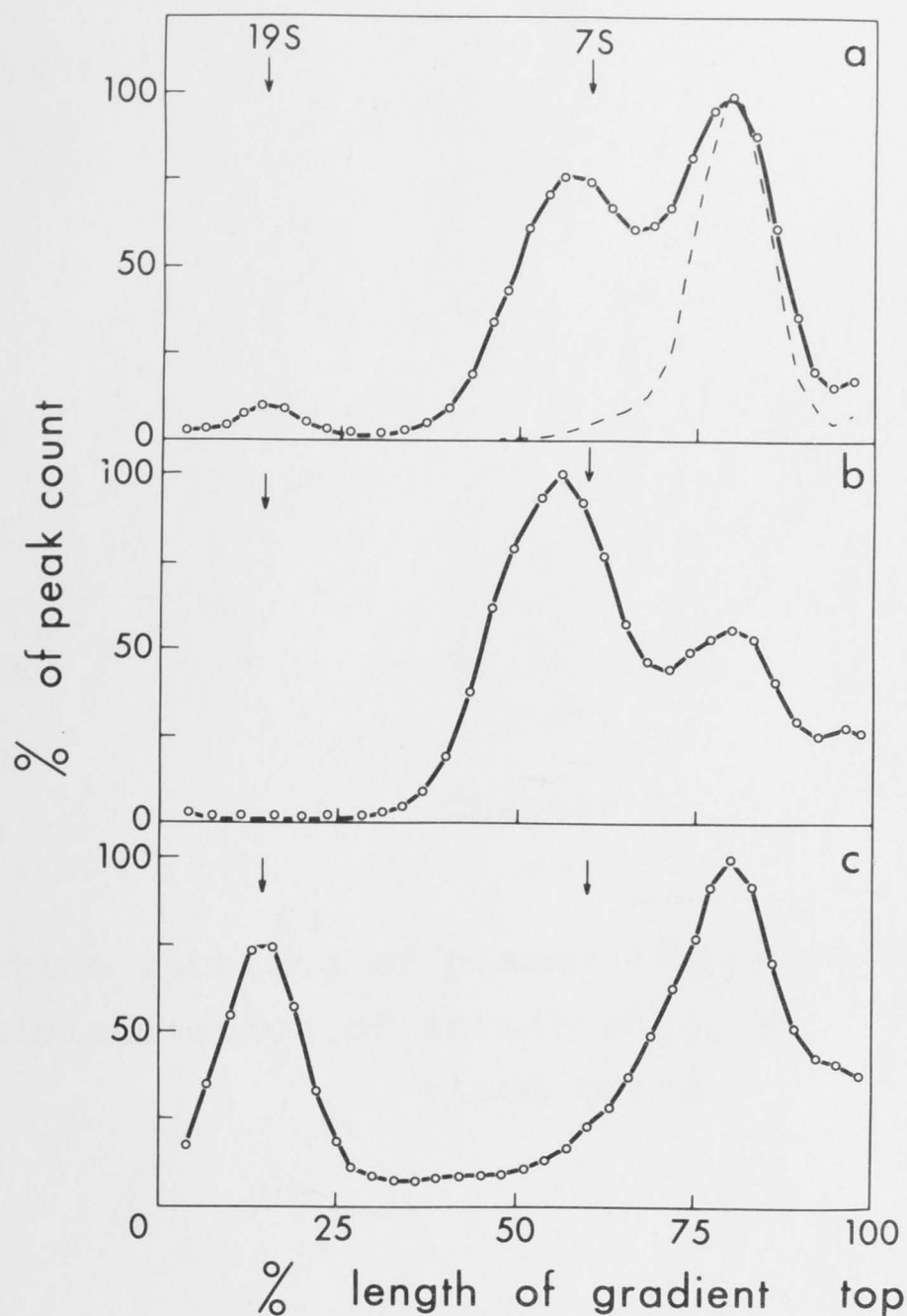


Figure 2.8: Sedimentation patterns of ^{125}I -labeled Fab (10 μl) specific for sheep (a) L chains, (b) γ chains and (c) μ chains, after incubation (37°C , 60 min) of each with 100 μl sheep serum.

Each sample was sedimented through gradients of sucrose (10 - 30 %, w/v) dissolved in DBSS containing 0.05 % NP40 and 15 mM NaN_3 . The broken line in (a) indicates the sedimentation position of untreated Fab, and the positions of 7S IgG and 19S IgM are shown. Radioactive material sedimenting at 3.5S after reaction with sheep serum is labeled Fc piece and inactive labeled Fab. Individual counts are plotted as a percentage of the peak count, which was 2623 cps (a), 2452 cps (b) and 1905 cps (c).

3.1 Introduction

3.2 Experimental results

3.3 Discussion

3.4 Summary

3.1 Introduction

The characterization of surface (plasma) membrane components, such as receptors for antigen, which may also occur intracellularly requires that components on the external surface of the cell be distinguished from those present intracellularly. This is particularly important as plasma membrane components, which constitute only a small proportion of the total cell mass, are easily lost by subcellular

Chapter 3

fractionation procedures. Such a distinction is possible using

chemical reagents which react specifically with components on the plasma membrane. Such reagents must be non-toxic, non-ionic, and must not alter the properties of the components in charge, conformation and function. The labeled components are minimal.

A number of chemicals including 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (Maddy, 1968), 7-chloromercuribenzenesulphonic acid (Van Steveninck, Wood and Rothstein, 1965; Sutherland, Rothstein and Wood, 1967; Tsan and Berlin, 1971), sulphamyl acid diazonium salt (Gardan and Watanabe, 1968; Berg, 1969; Sender, Gardan and Berg, 1971) and formylmethionylsulphonic methyl phosphate (Bretscher, 1971) have been used for this purpose with varying degrees of success. These reagents, all sulphonic acid derivatives, do not enter cells because of their acidic properties. Despite their proven value, however, they are not ideal. Firstly they react with and block protein side-chain residues, particularly sulphhydryl or amino groups and to a lesser extent histidine and tyrosine residues. These residues are frequently necessary for functional activity such as enzymic catalysis or vectorial transport. Secondly, they are of necessity highly acidic and consequently create new ionic environments in the region of substitution. This may cause conformational changes in the labeled protein. Thirdly, they are relatively large and may sterically interfere with functional properties of the

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3.1 Introduction

The characterization of surface (plasma) membrane components, such as receptors for antigen, which may also occur intracellularly requires that components on the external surface of the cell be distinguished from those present intracellularly. This is particularly important as plasma membrane components, which constitute only a small proportion of the total cell mass, are often solubilized by subcellular fractionation procedures. Such a distinction is possible using chemical markers which react covalently and exclusively with the plasma membrane. These reagents should ideally be small, nonionic, and react with membrane components such that changes in charge, conformation and function of the labeled components are minimal.

A number of chemicals including 4-acetamido-4'-isothio-cyanostilbene-2,2'-disulphonic acid (Maddy, 1964), *p*-chloromercuribenzenesulphonate (Vansteveninck, Weed and Rothstein, 1965; Sutherland, Rothstein and Weed, 1967; Tsan and Berlin, 1971), sulphanilic acid diazonium salt (Pardee and Watanabe, 1968; Berg, 1969; Bender, Garan and Berg, 1971) and formylmethionylsulphone methyl phosphate (Bretscher, 1971) have been used for this purpose with varying degrees of success. These reagents, all sulphonic acid derivatives, do not enter cells because (it is presumed) of their acidic properties. Despite their proven value, however, they are not ideal. Firstly they react with and block protein side-chain moieties, particularly sulphydryl or amino groups and to a lesser extent histidine and tyrosine residues. These residues are frequently necessary for functional activity such as enzymic catalysis or vectorial transport. Secondly, they are of necessity highly acidic and consequently create new ionic environments in the region of substitution. This may cause conformational changes in the labeled protein. Thirdly, they are relatively large and may sterically interfere with functional properties of the

protein. Functional changes resulting from substitution have been observed (Vansteveninck, Weed and Rothstein, 1965; Sutherland, Rothstein and Weed, 1967; Tsan and Berlin, 1971; Pardee and Watanabe, 1971).

Alternatively, the radioisotopes ^{125}I or ^{131}I can be used to label proteins. Iodine is superior in several ways to the abovementioned compounds: (a) It reacts only with tyrosine residues if catalyzed by lactoperoxidase (Marchalonis, 1969) and also (but to a lesser extent) with histidine residues if chemically oxidized using chloramine-T (Lamoureux, Carnegie and McPherson, 1967). Iodination of tyrosine occurs by substitution of hydrogen(s) *ortho* to the phenolic hydroxyl, only slightly altering its pK_a . (b) Steric interference is minimal as no more than two iodine atoms are substituted into the tyrosyl ring. (c) No new ionic charges are introduced to labeled proteins as iodine is not ionized when covalently-linked to an aromatic carbon. (d) Trace-labeling can be employed using isotopically-pure radioiodine. Thus changes in the ionic, conformational and functional properties of radioiodinated proteins should be minimal. Oxidation, especially of cysteine and methionine residues, is the major concern during protein iodination but can be minimized to levels which are in most cases acceptable (Sonoda and Schlamowitz, 1970; Parish and Stanley, 1972).

Consequently intact cells were radioiodinated to determine whether their plasma membrane components could be labeled exclusively. Chloramine-T oxidation was employed as this method was used routinely to label soluble proteins. The rationale was that surface proteins were more likely than intracellular proteins to be iodinated, *especially* if chloramine-T did not readily enter the cells. This could be expected because chloramine-T (the sodium salt of N-chloro-*p*-toluene-sulphonamide) contains a sulphonic acid group. Sheep and chicken red blood cells were chosen for this study because of their ready availability, the ease of isolation of their plasma membranes, and their content of a highly coloured intracellular protein whose release provides a rapid and sensitive measure of membrane damage.

3.2 Experimental results

3.2.1 Iodination of sheep red blood cells

3.2.1.1 Effect of temperature, chloramine-T concentration and reaction time on the iodination of intact SRBC

To determine the quantity of chloramine-T required to iodinate intact SRBC, cells were washed in DBSS and reacted with ^{125}I (total iodide = $1 \times 10^{-7}\text{M}$) at 0°C or 25°C for a standard time (15 min) at different concentrations of the oxidant (Fig 3.1). Iodination was insignificant at $\leq 10^{-6}\text{M}$ chloramine-T, but increased markedly at higher concentrations. At 1×10^{-4} , 1×10^{-3} and $1 \times 10^{-2}\text{M}$ chloramine-T respectively, SRBC at 25°C were labeled 1.0, 2.2 and 4.1 times more heavily than at 0°C . Except for those cells exposed to $1 \times 10^{-2}\text{M}$ chloramine-T, no haemolysis of labeled SRBC was evident after standing overnight at 0°C . Chloramine-T was routinely used at 1×10^{-4} to $1 \times 10^{-3}\text{M}$ in subsequent work. This concentration range was chosen because iodination was poor at lower concentrations, while at higher concentrations oxidative side-reactions were more likely.

The time course of SRBC labeling is shown in Fig 3.2. Two rates are evident. Iodination occurred rapidly and was virtually complete within 1-2 min. at either 0°C or 25°C . After this initial reaction, however, the cells continued to become labeled at a slower, temperature-dependent, linear rate (0°C : 0.36 atoms iodine/SRBC/min; 25°C : 1.51 atoms/SRBC/min.). As metabolic processes are inhibited and membrane fluidity is decreased markedly at 0°C , this continued labeling was most likely the result of facilitated diffusion or active transport of iodine or iodide into the cells. Furthermore, the iodination reaction consumed only 0.67 % of the available iodide and most of the chloramine-T (present in 2000-fold molar excess over iodide) may therefore have been consumed in reactions other than iodination. Chloroform/methanol extraction of total lipids from whole labeled SRBC (using the method of Folch, Lees and Stanley, 1957) removed 13 % of cell-associated radioactivity, indicating that mainly nonlipid material, probably protein, was being iodinated.

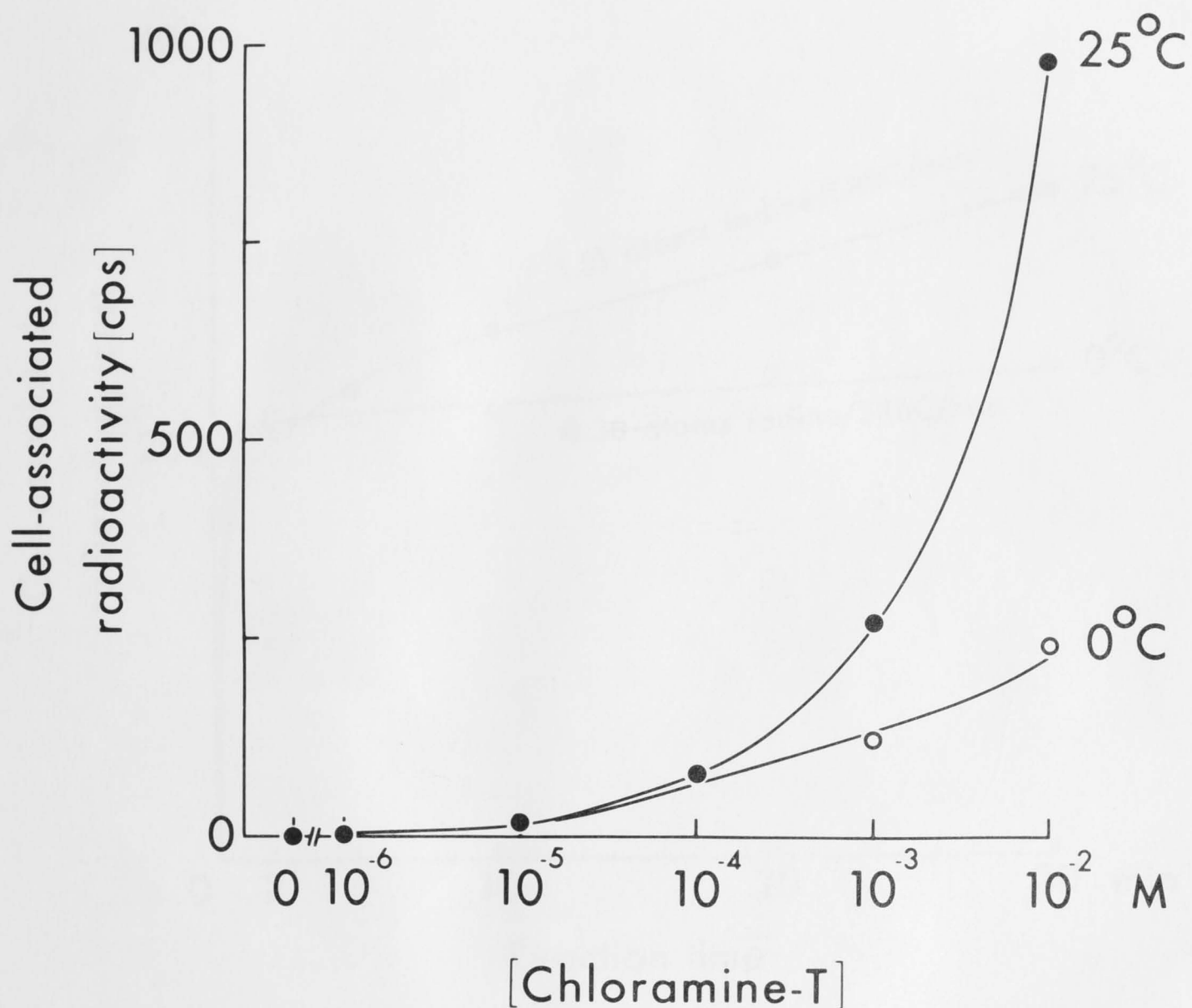


Figure 3.1: Iodination of intact SRBC at different concentrations of chloramine-T.

Each reaction mixture (140 μ l) contained 4×10^9 SRBC/ml, 1×10^{-7} M iodide (45 μ Ci/ml) and chloramine-T as indicated in DBSS. After reacting at either 0°C or 25°C for 15 min, unreacted chloramine-T was reduced by addition of an equimolar quantity of sodium meta-bisulphite dissolved in 20 μ l DBSS. Each mixture was then diluted with 3 ml ice-cold DBSS/1 mM KI and after centrifugation (800xg, 10 min), the cells were washed 5 times with 3 ml aliquots of the same buffer before being suspended in 3 ml and left overnight at 0°C. They were then sedimented and assayed for radioactivity.

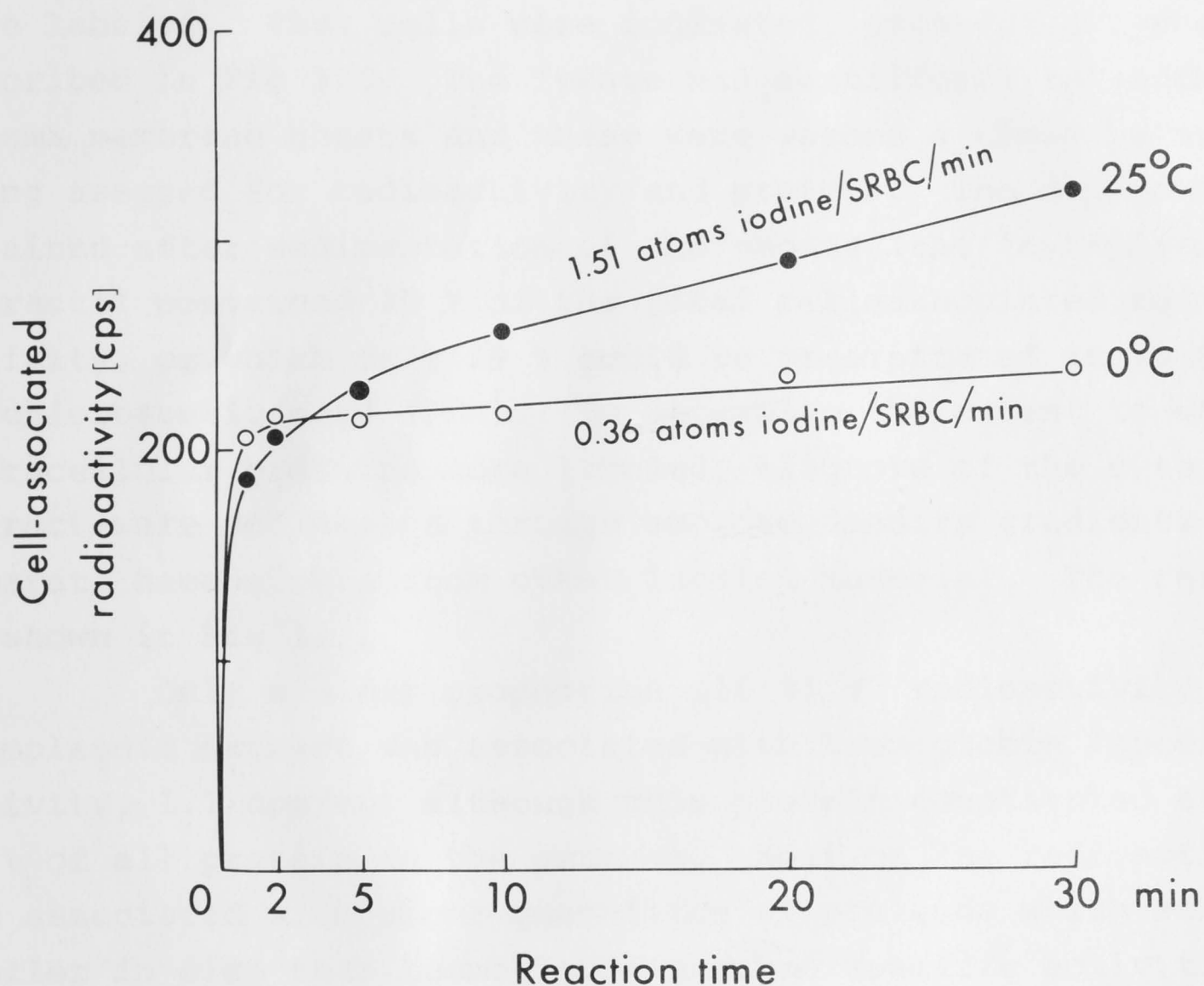


Figure 3.2: Time course of iodination of intact SRBC.

SRBC were iodinated at 0°C and at 25°C. Each reaction mixture (800 μ l) contained 4×10^9 SRBC/ml, 1×10^{-7} M iodide (45 μ Ci/ml) and 2×10^{-4} M chloramine-T in DBSS. Samples (100 μ l) were removed at the indicated times, immediately mixed with 100 μ l of 2×10^{-4} M $\text{Na}_2\text{S}_2\text{O}_5$ at 0°C and diluted with 2 ml of DBSS containing 1 mM KI. The cells were washed 6 times at 0°C before being assayed for radioactivity.

3.2.1.2 Distribution of radioactivity within iodinated SRBC

Having established that intact SRBC could be iodinated, it was important to determine which components of the cell were labeled. Thus cells were iodinated, washed and lysed as described in Fig 3.3. The lysate was centrifuged to sediment plasma membrane ghosts and these were washed 4 times before being assayed for radioactivity and protein. The supernatant obtained after sedimentation of the ghosts (the 'cytoplasmic extract') contained 29 % of the total cell-associated radioactivity, of which only 29 % could be precipitated in 10 % (w/v) trichloroacetic acid (TCA). To determine the extent to which intracellular proteins were labeled, aliquots of the cytoplasmic extract were sedimented through sucrose density gradients to separate haemoglobin from other labeled material. The result is shown in Fig 3.3.

Only a minor proportion (16 %) of radioactivity in the cytoplasmic extract was associated with haemoglobin (specific activity, 1.7 cps/mg) although this protein constituted at least 98 % of all protein in the extract. Most of the radioactivity was associated with minor quantities of proteins which were smaller in size than haemoglobin and had specific activities of > 200 cps/mg. These proteins were therefore labeled at least 118 times more heavily than haemoglobin, although they were exceeded approximately 100-fold (wt/wt) by the latter protein. As purified haemoglobin was easily iodinated under conditions identical to those used to label intact SRBC, these smaller proteins present in the cytoplasmic extract were almost certainly solubilized plasma membrane components. Other radioactive material (30 % of total cytoplasmic label) which remained above the gradients was probably unreacted iodide taken up by the cells during iodination.

The distribution of radioactivity within the various cell fractions is shown in Table 3.1. The plasma membrane ghosts, which comprised only 2 % of the total cell protein, contained 71 % of the cell-associated radioactivity and had a specific activity of 924 cps/mg protein. This represented a 34-fold enrichment over whole cells. In contrast, the cytoplasmic extract contained almost all the cell protein but

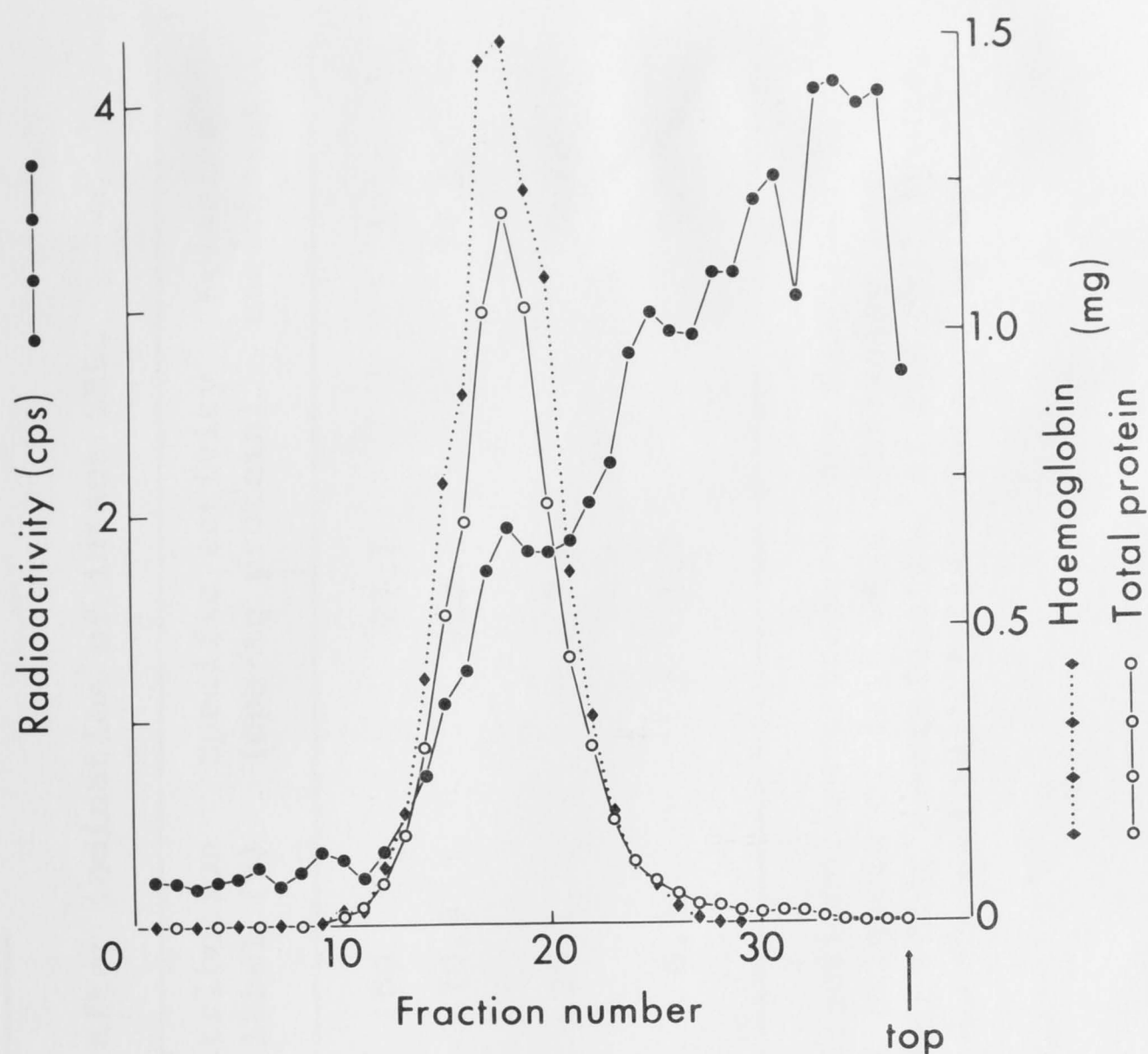


Figure 3.3: Distribution of haemoglobin, total protein and radioactivity after sedimentation of the cytoplasmic extract of ^{125}I -labeled SRBC.

The reaction mixture (1.1 ml) contained 5×10^9 SRBC/ml, $< 1 \times 10^{-7}$ M carrier-free $\text{Na} [^{125}\text{I}]$ (150 $\mu\text{Ci/ml}$) and 1×10^{-4} M chloramine-T in DBSS. After 5 min at 25°C , 100 μl of 1.1 mM $\text{Na}_2\text{S}_2\text{O}_5$ was added followed by 3.5 ml of DBSS/1 mM KI. The cells were sedimented, washed 6 times with 3.5 ml aliquots of DBSS/1 mM KI at 0°C and then lysed by suspension in 5 ml of 1/10 DBSS (DBSS diluted 1/10 with water) containing 1 mM KI. After 60 min at 0°C , the lysate was centrifuged (SW50.1 rotor; 35,000 rpm, 60 min) to sediment plasma membrane 'ghosts'. The supernatant was removed and recentrifuged to remove residual ghosts. The resulting red supernatant was termed the 'cytoplasmic extract'.

A 0.2 ml aliquot of the cytoplasmic extract was sedimented through each of two identical 10-30 % (w/v) sucrose gradients containing DBSS and 15 mM azide (SW50.1 rotor; 45,000 rpm, 29 h, 7°C). Fractions (0.13 ml) were collected by tube puncture. Corresponding fractions from each gradient were pooled and diluted with 0.75 ml DBSS. Each fraction was then assayed for radioactivity, haemoglobin and total protein. Haemoglobin was calculated from $A_{430, 1 \text{ cm}}$ using a molar extinction coefficient of 128,000 (Sidwell, Munch, Guzman Barron and Hogness, 1938). Total protein was estimated with Folin's reagent using bovine serum albumin as a standard.

Table 3.1

Distribution of radioactivity after iodination of intact SRBC

Fraction	Protein (mg)	Radioactivity (cps)	Distribution of label (%)	Specific activity (cps/mg protein)	Relative enrichment
Total SRBC	96	2605	100	27.1	1
Ghosts	2.0	1847	70.9	923.5	34.1
Cytoplasmic extract	100	758	29.1	7.6	0.28
Purified haemoglobin	100	165	6.3	1.7	0.06

The conditions of iodination and subcellular fractionation procedures are described in Fig. 3.3. Ghosts were washed 4 times with 5 ml aliquots of 1/10 DBSS containing 1 mM KI. Cytoplasmic extract was the supernatant obtained after centrifugation of the cell lysate (SW50.1 rotor; 35,000 rpm, 60 min). Protein was determined with Folin's reagent using bovine serum albumin as a standard.

only 29 % of the cell-associated radioactivity and had a specific activity of 7.6 cps/mg protein. As mentioned above, however, little of this radioactivity was associated with haemoglobin.

3.2.1.3 Isolation of plasma membranes from ^{125}I -labeled SRBC by equilibrium density centrifugation

Intact SRBC were iodinated, washed and lysed by hypotonic shock as described in Fig 3.4. The lysates were then layered over sucrose density gradients and subjected to equilibrium density centrifugation. Lysis only, as well as lysis followed by Dounce homogenization, produced a single white, opaque band of mean density 1.142 g/cc which contained much of the original cell-associated radioactivity. Fractions 22-31 (density < 1.08) contained essentially all the haemoglobin, but less than 30 % of the total radioactivity. Cells disrupted by hypotonic shock contained traces of aggregated material which was eliminated by homogenization. Such treatment resulted in 71 % of the radioactivity banding between density 1.135 and 1.150. This proportion is identical to that found associated with washed ghosts (section 3.2.1.2).

Fig 3.5 illustrates the density banding pattern of radioactivity and of cell surface antigenic determinants after centrifugation of ultrasonicated ^{125}I -labeled SRBC. The distribution of radioactivity was similar to that of Fig 3.4 and nearly identical to that of cell surface antigenic determinants. Thus the labeled material of density 1.142 was almost certainly the SRBC plasma membrane.

3.2.2 Iodination of chicken red blood cells

Chicken red cells were selected as a second cell system to determine whether their plasma membranes, like those of SRBC, would be preferentially labeled by nonenzymic iodination of intact cells. Because they are nucleated, however, CRBC are structurally more complex than SRBC. It has been calculated from electron microscope measurements on CRBC that 54.2 % of the total cellular membrane material is constituted by plasma membranes, 45.6 % by nuclear membranes and < 1 % by other membranes such as those from mitochondria, lysosomes or

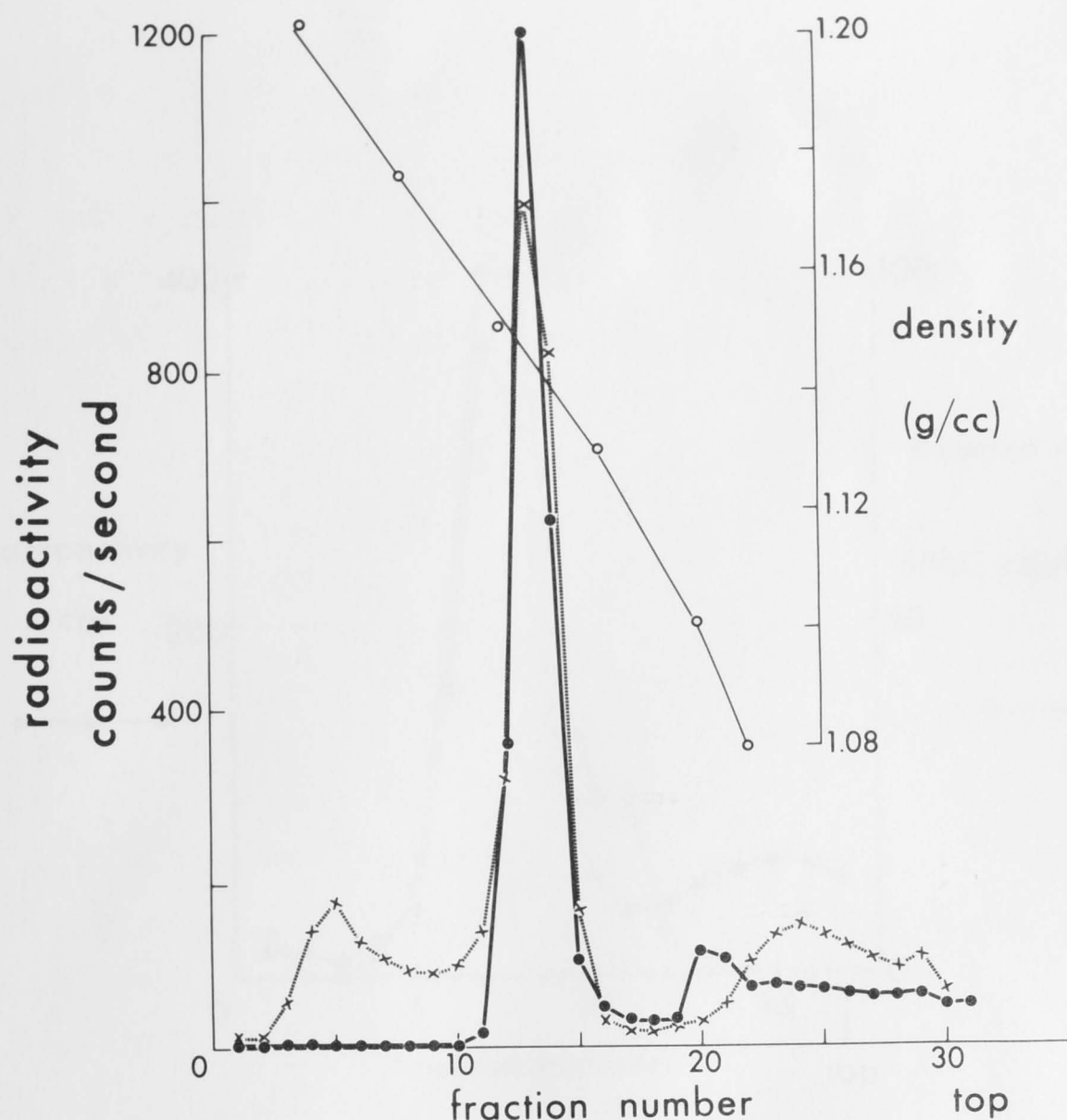


Figure 3.4: Equilibrium density centrifugation of ^{125}I -labeled SRBC after hypotonic lysis and dounce-homogenization.

Cells were labeled in a 210 μl reaction volume containing, in DBSS, 8×10^9 SRBC/ml, $< 2 \times 10^{-7}$ M ^{125}I (carrier-free, 400 $\mu\text{Ci}/\text{ml}$) and 5×10^{-4} M chloramine-T. After 5 min at 25°C , 5 μl 20 mM $\text{Na}_2\text{S}_2\text{O}_5$ and then 5 ml DBSS/1 mM KI were added. The cells were washed 5 times with 10 ml aliquots of DBSS/1 mM KI and then suspended in a final volume of 9 ml.

Two 3 ml aliquots of the labeled SRBC were centrifuged ($800 \times g$, 10 min) and each cell pellet was resuspended at 0°C in 10 ml 5 mM MgCl_2 /20 mM Tris-HCl, pH 7.4. The second suspension only was dounce-homogenized (60 strokes). Each suspension was then layered over a 20-55 % (w/v) sucrose density gradient containing 5 mM MgCl_2 /20 mM Tris-HCl, pH 7.4 and centrifuged for 3 h (SW25.1 rotor; 24,000 rpm, 4°C). The gradients were fractionated by tube puncture and the refractive index and radioactivity of each fraction was measured.

	Treatment	Total (cps)	Radioactivity	
			Pellet*	#11-15 (incl.)
			(%)	(%)
X---X	hypotonic lysis	4481	5.8	56.5
●—●	lysis + dounce	3604	3.5	70.7

* Material which sedimented through the gradient to the bottom of the tube.

The density of the major peak (fraction 13) was 1.142 g/cc. Fractions 22-31 were deep red in colour and contained essentially all the haemoglobin.

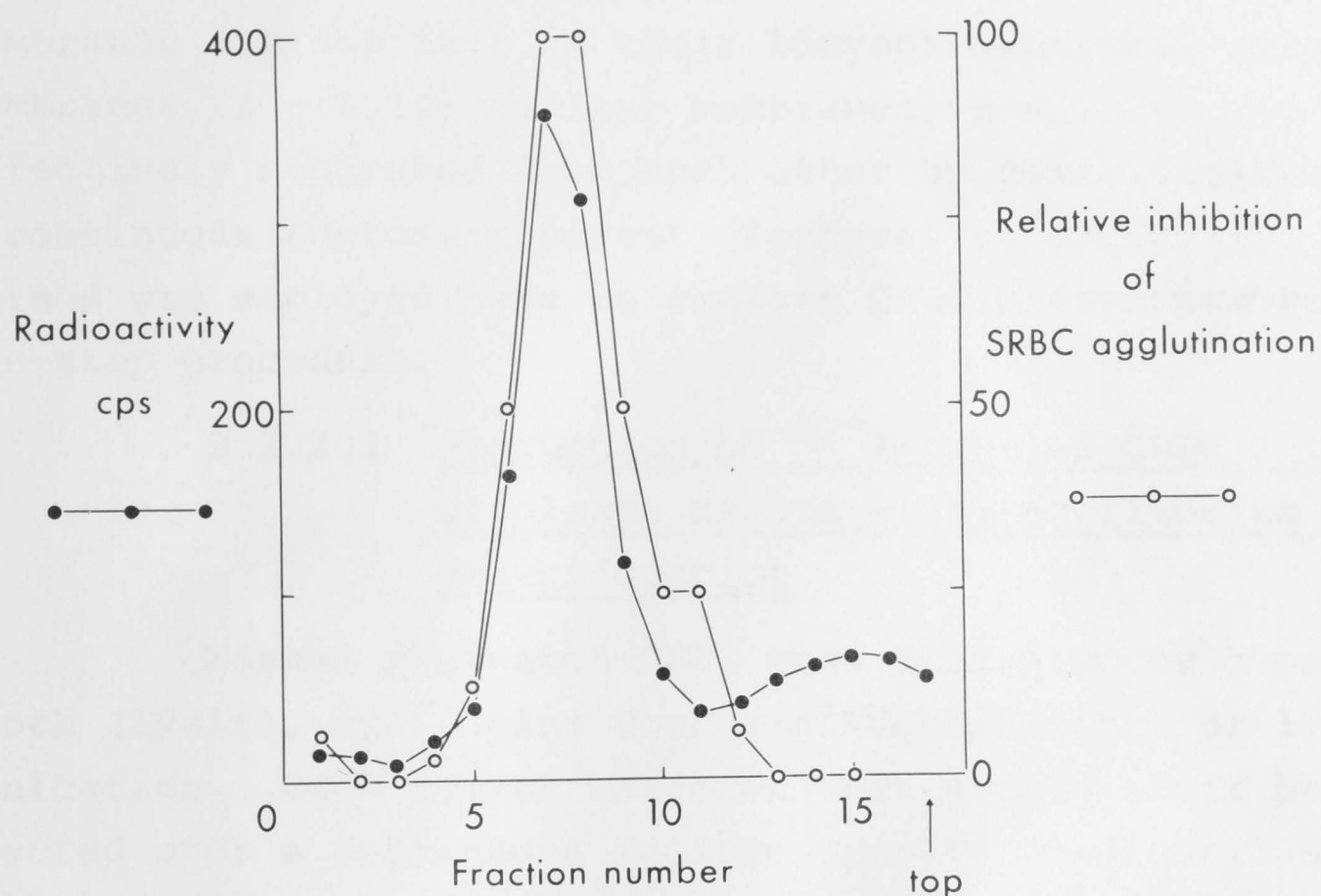


Figure 3.5: Equilibrium density centrifugation of ^{125}I -labeled SRBC after ultrasonic rupture: distribution of surface antigens.

SRBC were labeled and washed as described in Fig. 3.4. They were then mixed with 0.9 ml of packed unlabeled SRBC (1.6×10^{10} cells), suspended in 10 ml of 5 mM MgCl_2 /0.1 M NaCl /50 mM Tris-HCl , pH 7.5, sonicated (30 seconds) and 5 ml layered over a 25-50 % (w/v) sucrose gradient made with the above buffer. After centrifugation (SW25.3 rotor; 22,000 rpm, 18 h, 5°C), 1 ml fractions were collected by tube puncture and tested for radioactivity and for their ability to inhibit agglutination of SRBC by anti-SRBC serum.

	Radioactivity (cps)	Distribution of label (%)
Total applied to gradient	1522	100
Pellet (sediment)	57.8	3.8
Fractions 5-10 (inclusive)	1040	68.3

endoplasmic reticulum (Zentgraf, Deumling, Jarasch and Franke, 1971). Thus, CRBC can be regarded as containing essentially only two kinds of membranes, plasma and nuclear. These membranes are distinct in their buoyant densities (plasma membranes, $\rho = 1.14$; nuclear membranes, $\rho = 1.20$) and can be effectively separated from each other by centrifugation through a continuous sucrose gradient (Zentgraf *et al.*, 1971). This method was employed here to isolate CRBC plasma membranes in a one-step procedure.

3.2.2.1 Disruption of ^{125}I -labeled CRBC : isolation of plasma membranes by equilibrium density centrifugation

Washed iodinated CRBC were disrupted by hypotonic shock (lysis), lysis plus Dounce homogenization, or lysis plus sonication. Each lysate was then centrifuged after being layered over a continuous sucrose density gradient. As seen from Fig 3.6, the distribution of radioactivity obtained depended on the method used to fragment the cells. For cells ruptured by hypotonic shock, 88 % of the cell-associated radioactivity sedimented completely through the gradient. The other 12 % remained in the haemoglobin-rich fractions overlying the gradient. Dounce homogenization reduced to 76 % the proportion of radioactivity associated with the sediment and caused the appearance of an opaque, cream-coloured band of radioactive material with a mean peak density of 1.163 g/cc. Sonication further reduced (to 44 %) the proportion of radioactivity associated with the sediment. Most of this material (27 % of the cell-associated radioactivity) banded between density 1.14-1.18 and the remainder (17 % of total) was dispersed through the gradient above this band.

To determine whether the labeled material of density 1.163 was the plasma membrane, fractions were tested for their content of CRBC surface antigenic determinants. The distribution of these determinants in the gradient after centrifugation of sonicated labeled cells is shown in Fig 3.7. The pattern resembled closely that of radioactivity, with most of the determinants concentrated between density 1.140 - 1.18. No determinants were detected in the (cytoplasmic) fractions overlying the gradient. The association of radioactivity and surface

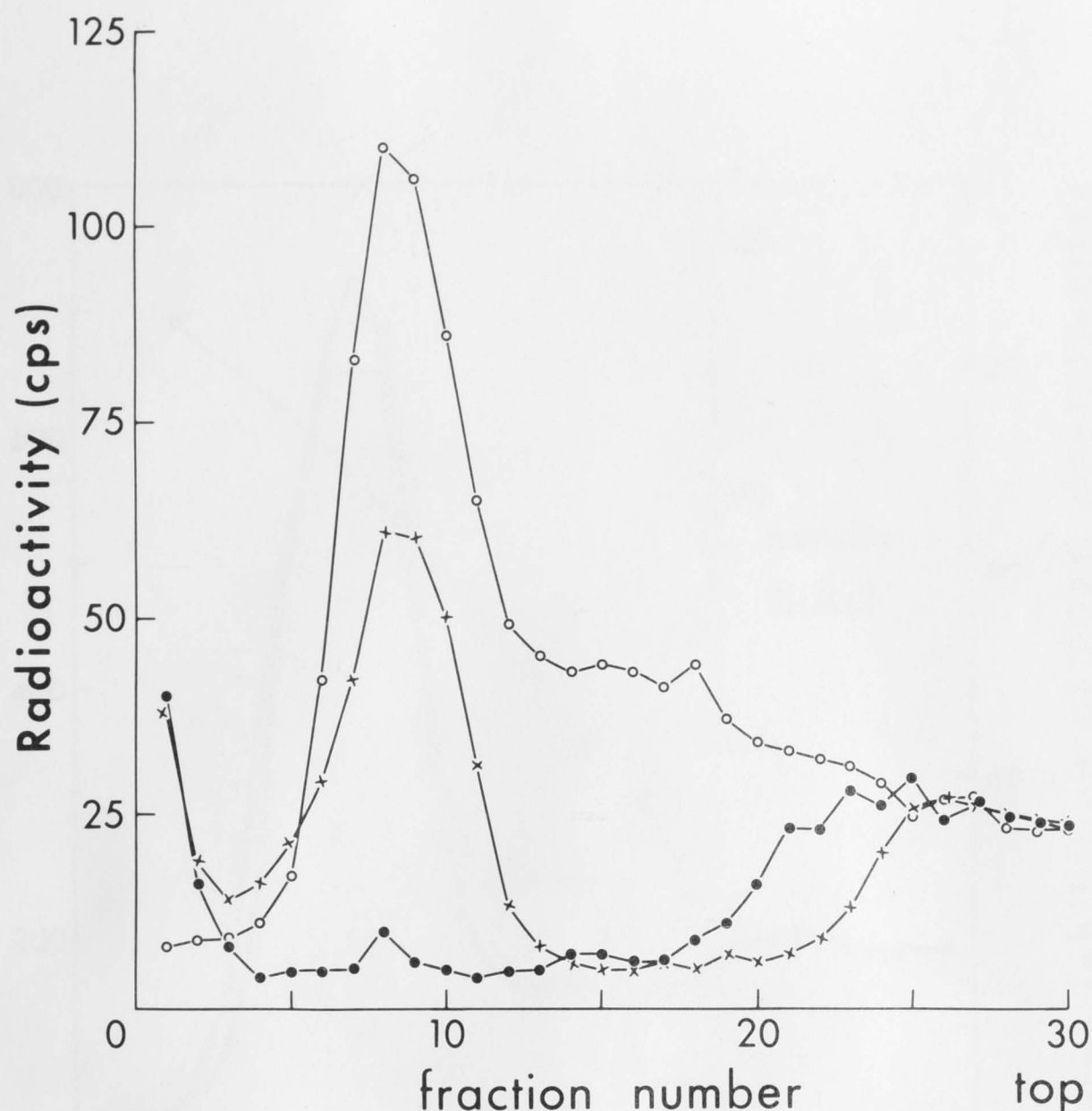


Figure 3.6: Equilibrium density centrifugation of ^{125}I -labeled CRBC after hypotonic lysis, dounce-homogenization or ultrasonic fragmentation.

CRBC were iodinated for 5 min at 25°C in a reaction volume of $210\ \mu\text{l}$ containing 4×10^9 cells/ml, $< 2 \times 10^{-7}\ \text{M}$ Na^{125}I (carrier-free, $400\ \mu\text{Ci/ml}$) and $5 \times 10^{-4}\ \text{M}$ chloramine-T. After addition of $5\ \mu\text{l}$ $20\ \text{mM}$ $\text{Na}_2\text{S}_2\text{O}_5$ and $5\ \text{ml}$ DBSS/ $1\ \text{mM}$ KI, the cells were washed 5 times with $10\ \text{ml}$ aliquots of DBSS/ $1\ \text{mM}$ KI. They were then divided into 3 equal fractions a, b and c, sedimented and each cell pellet was resuspended in $10\ \text{ml}$ $5\ \text{mM}$ MgCl_2 / $20\ \text{mM}$ Tris-HCl, pH 7.4. Fraction (a) was given no further treatment, fraction (b) was dounce-homogenized (50 strokes) and fraction (c) sonicated for 60 seconds. Each preparation was then layered over a 20-55 % (w/v) sucrose gradient containing $5\ \text{mM}$ MgCl_2 / $20\ \text{mM}$ Tris-HCl, pH 7.4 and centrifuged for 3 h (SW25.1 rotor; $24,000\ \text{rpm}$, 4°C). Fractions were collected by tube puncture and assayed for radioactivity.

Cell treatment	Radioactivity		
	Total (cps)	Pellet (%)	Fractions 5-13 inclusive (%)
●—● Lysis	3208	87.6	1.7
X—X Lysis + homogenization	2995	75.9	11.3
O—O Lysis + sonication	2244	43.5	27.4

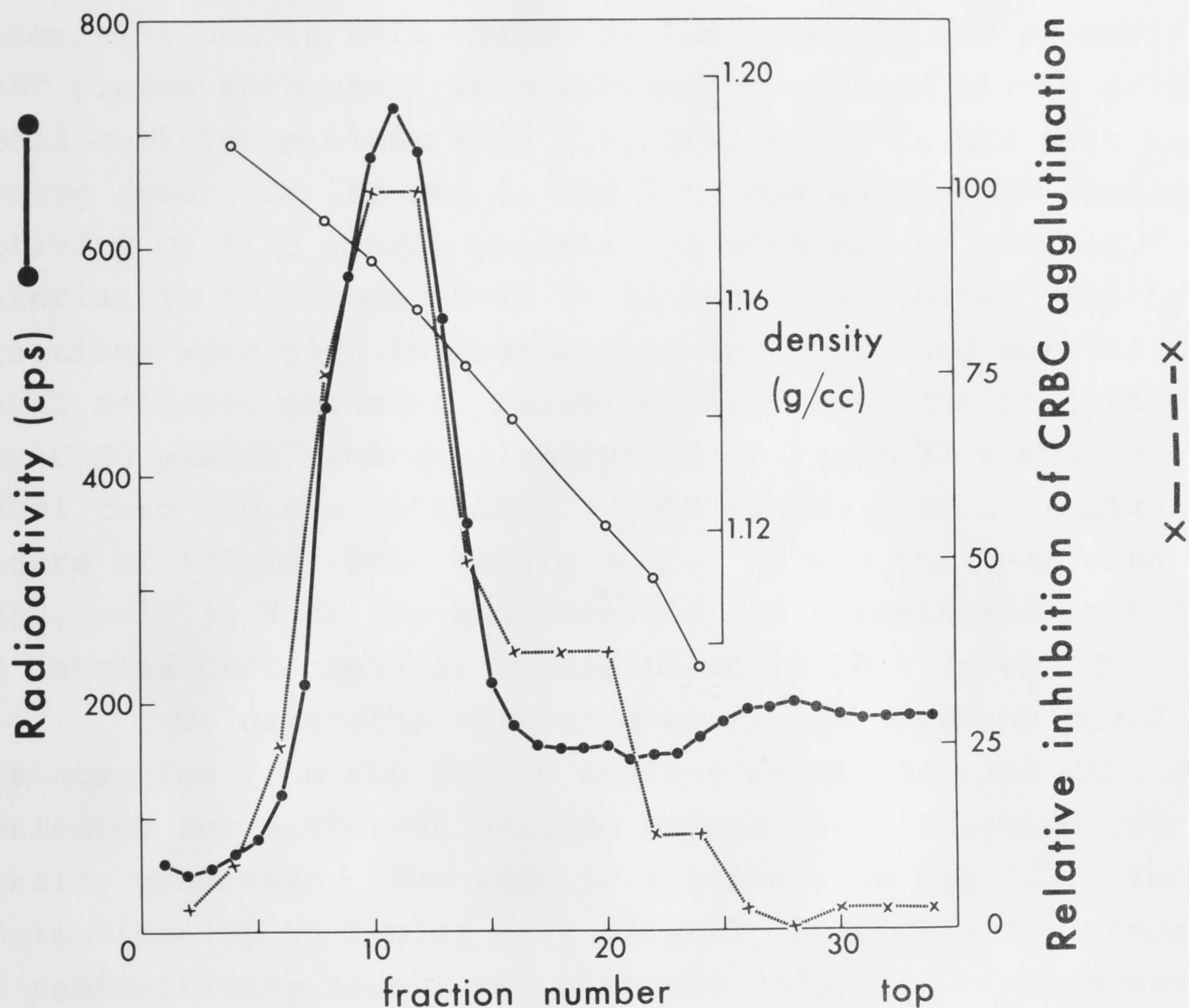


Figure 3.7: Equilibrium density centrifugation of sonicated ^{125}I -labeled CRBC: distribution of surface antigenic determinants.

CRBC were labeled and washed as described in Fig. 3.6. They were then mixed with 8 ml of 10 % (v/v) unlabeled CRBC (7×10^9 cells) and sedimented at $800 \times g$ for 10 min. The cell pellet (0.9 ml) was suspended in 6 ml of 5 mM MgCl_2 /0.1 M NaCl /50 mM Tris-HCl (pH 7.5), sonicated for 60 seconds, layered over a 25-50 % (w/v) sucrose gradient containing the above buffer and centrifuged for 18 h (SW 25.3 rotor; 22,000 rpm, 5°C). Fractions, 0.8 ml, were collected and assayed for radioactivity and refractive index. To determine the distribution of surface antigenic determinants, each fraction was also tested for its ability to inhibit agglutination of CRBC by anti-CRBC serum.

The sediment at the bottom of the gradient contained 30.3 % of the total radioactivity (11,730 cps) and material banding between density 1.15-1.18 contained 38.3 %. The mean peak density of the latter material was 1.163 g/cc.

antigens between density 1.14 - 1.18 indicated that the opaque material found in this region of the gradient was probably the CRBC plasma membrane. This material contained little of the total cellular protein (Fig 3.8). Material in the peak radioactive fractions (#5 and 6, Fig 3.8) had an average specific activity of 1175 cps/mg protein, in contrast to that of material in fractions 10-15 (< 31 cps/mg protein). The latter fractions were rich in haemoglobin and contained most of the total cellular protein. Based on these specific activities, the CRBC plasma membranes contained at least 37 times more label than did the cytoplasm. This compares to a similar figure of 121 for SRBC (Table 3.1). As was the case with SRBC, only 31 % of the radioactivity in cytoplasmic extracts of labeled CRBC could be precipitated in 10 % (w/v) TCA.

To determine whether more plasma membrane could be disentangled from the denser nuclear debris, labeled CRBC were sonicated for different periods before centrifugation over density gradients. The results are shown in Fig 3.9. Increased sonication (up to 5 min) only slightly decreased the proportion of radioactivity associated with the sediment (minimum was 19 % of total after 60 sec sonication). With prolonged sonication (1-5 min.), labeled plasma membrane material shifted to lower densities. This shift was found to be due to fragmentation of the membrane to pieces too small to sediment to their buoyant density within short periods of centrifugation. After prolonged centrifugation (Fig 3.10) up to 50 % of the total cell-associated radioactivity could be obtained in a narrow, well-defined plasma membrane band of peak density, 1.163.

3.2.2.2 Effect of buffer on the iodination of CRBC

After collection of fresh blood, CRBC were routinely washed and stored at 4°C in Alsever's solution. If this solution was not completely removed before iodinating the cells, the distribution of radioactivity within the labeled cells was found to differ significantly from that of cells labeled in DBSS. Radioactivity profiles obtained from cells which were labeled and washed in Alsever's solution, DBSS, or DBSS containing 15 mM NaN_3 before being sonicated and centrifuged over sucrose gradients, are shown in Fig 3.11.

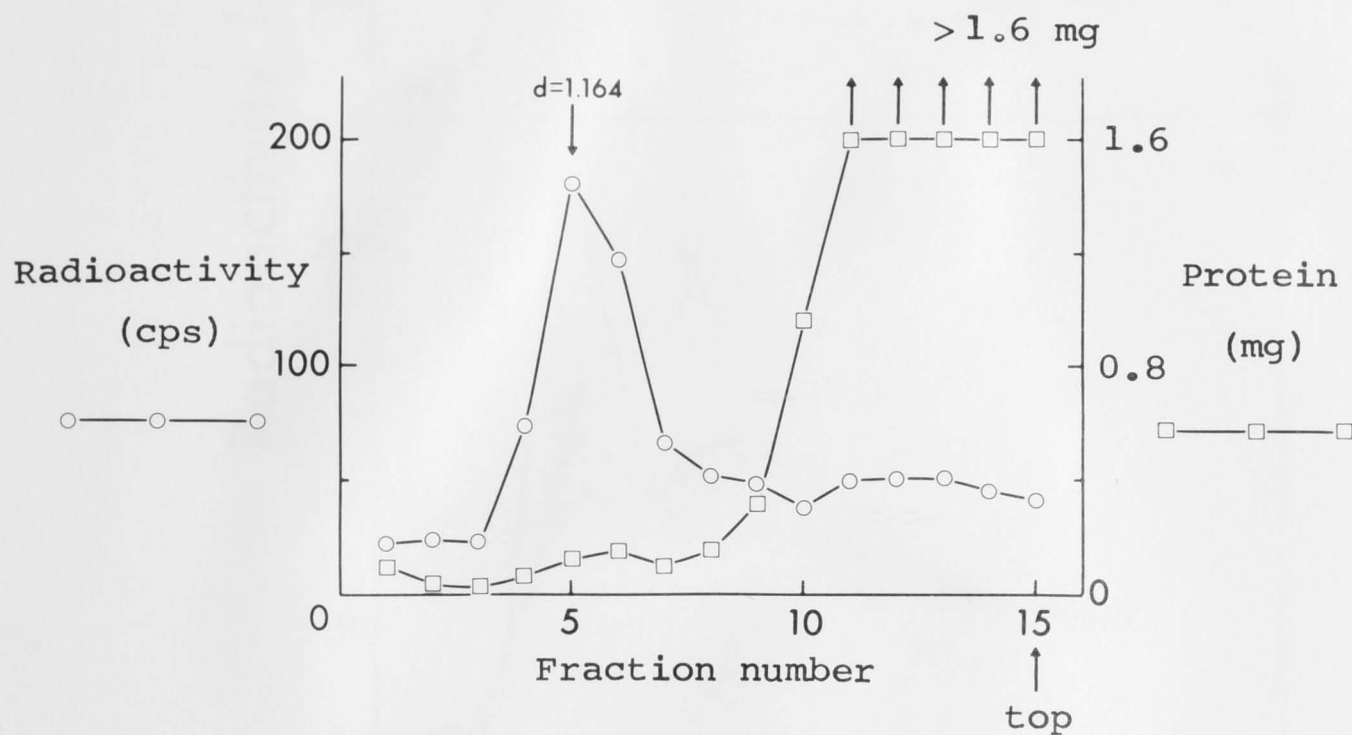


Figure 3.8: Distribution of protein after equilibrium density centrifugation of sonicated ^{125}I -labeled CRBC.

CRBC (1.7×10^9 cells) were labeled and washed as described in Fig. 3.6. They were resuspended in 5 ml of 5 mM MgCl_2 /20 mM Tris-HCl (pH 7.4), sonicated for 30 seconds, layered over a 25–50 % (w/v) sucrose gradient made with the same buffer and centrifuged for 5.5 h (SW25.3 rotor; 23,000 rpm, 5°C). Fractions, 1 ml, were collected and tested for radioactivity and for protein using Folin's reagent. The total radioactivity was 1563 cps of which 41 % was in the sediment.

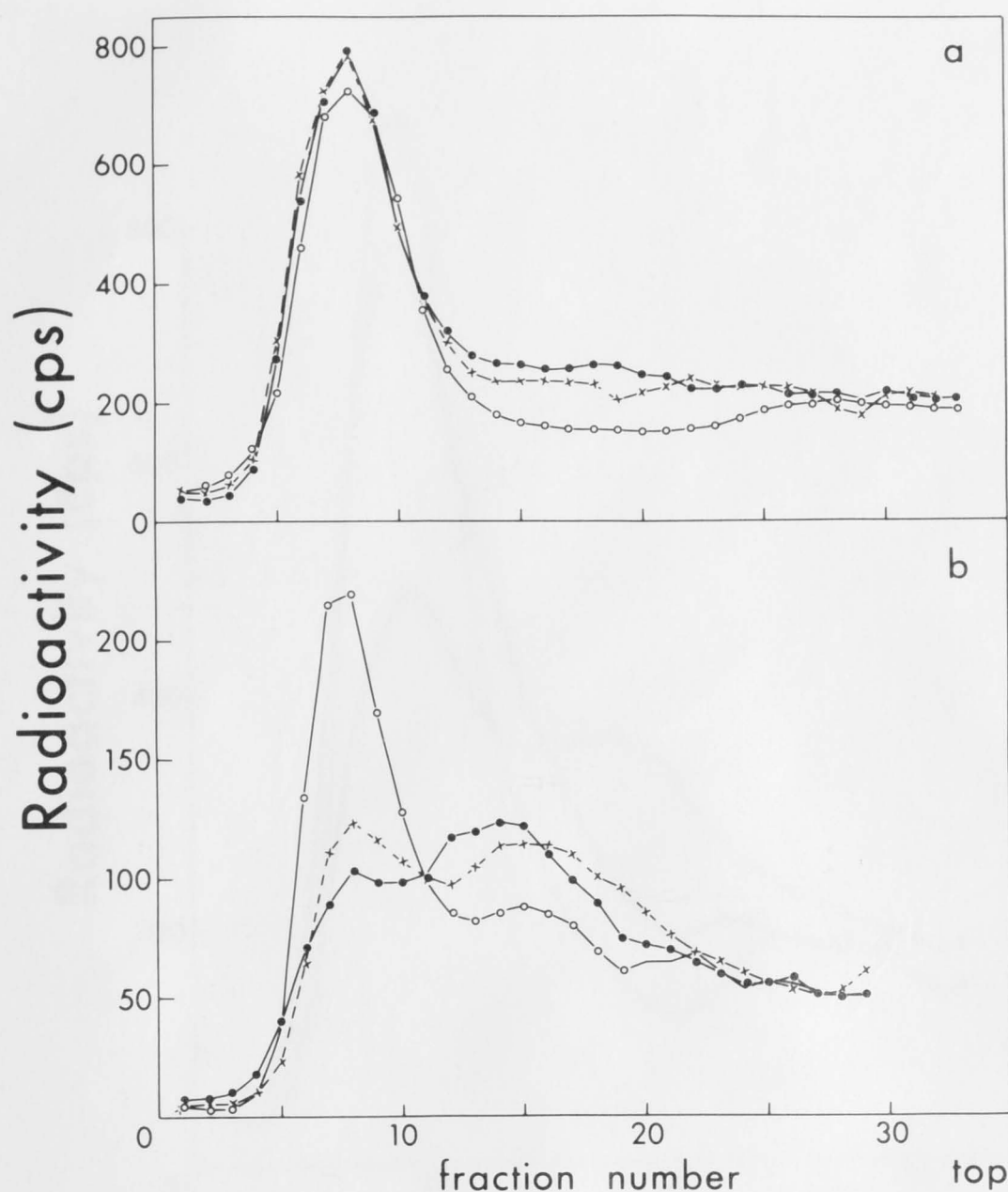


Figure 3.9: Effect of sonication time on the distribution in density gradients of radioactive material from ^{125}I -labeled CRBC.

- a) CRBC were labeled and washed as described in Fig. 3.6. They were divided into 3 equal fractions, centrifuged and each pellet was resuspended in 10 ml of 5 mM MgCl_2 /20 mM Tris-HCl, pH 7.4. They were then sonicated (see below), layered over 20-55 % (w/v) sucrose gradients containing the above buffer and centrifuged for 3 h (SW25.1 rotor; 24,000 rpm, 4°C). Fractions were assayed for radioactivity.
- b) CRBC were labeled as in (a) but sonicated for different times (see below).

			Radioactivity	
	Sonication time (seconds)	Total (cps)	Pellet (%)	Fractions 4-11 inclusive (%)
a)	O—O 15	11730	30.2	32.2
	X—X 30	12376	28.6	32.8
	●—● 45	12297	26.5	32.1
b)	O—O 60	2810	19.1	36.0
	X—X 180	2754	21.5	23.8
	●—● 300	2790	24.2	18.8

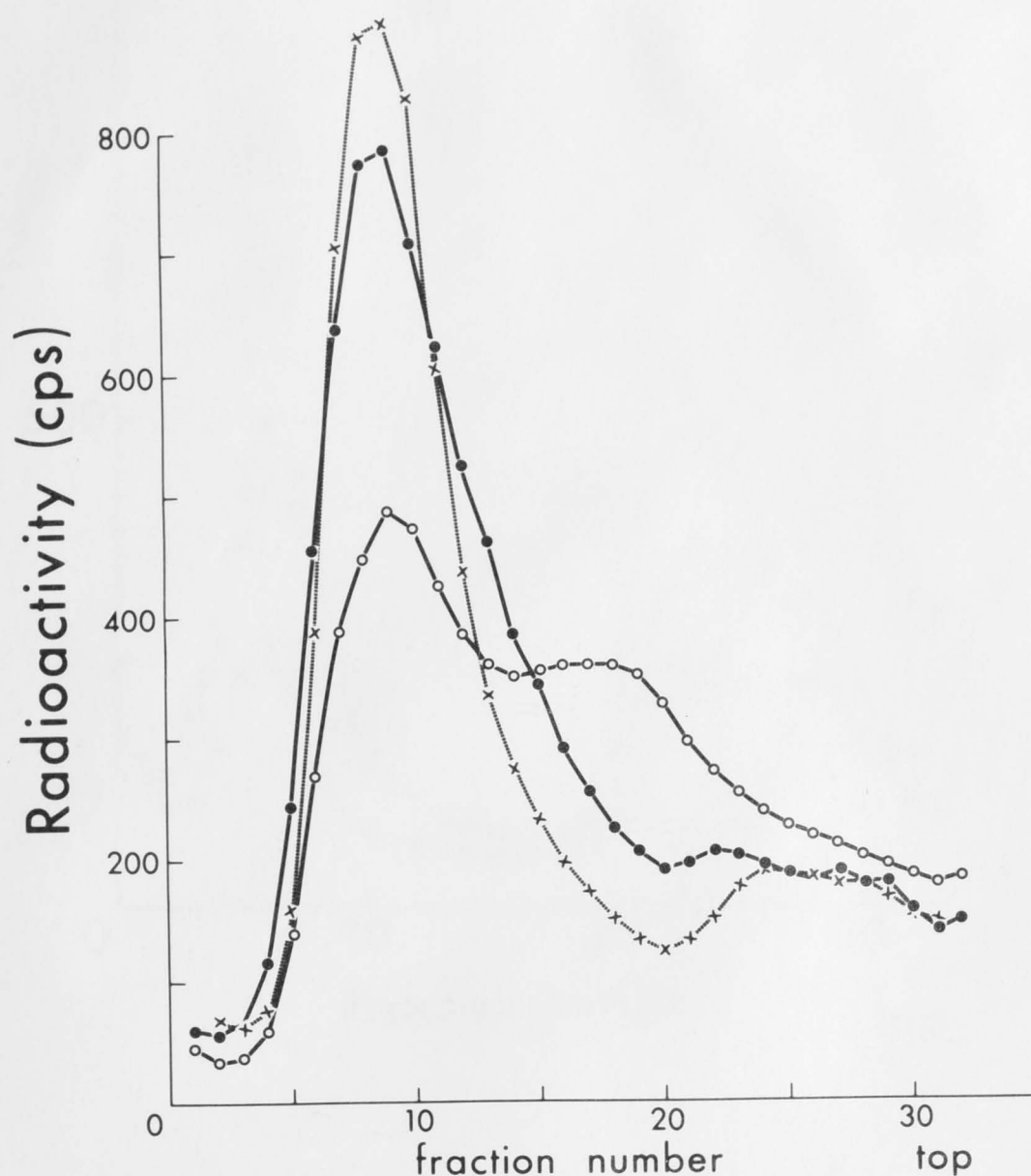


Figure 3.10: Distribution in density gradients of radioactive material from sonicated ^{125}I -labeled CRBC after prolonged centrifugation.

CRBC, labeled and washed as described in Fig. 3.6, were divided into 3 equal fractions and the cells sedimented. Each cell pellet was resuspended in 10 ml of 5 mM MgCl_2 /20 mM Tris-HCl, pH 7.4 and sonicated for 1 min (tube 1) or 3 min (tubes 2 and 3). Each sonicate was layered over a 20-50 % (w/v) sucrose gradient containing the sonication buffer and centrifuged for either 3 h (tube 3) or 13 h (tubes 1 and 2) (SW25.1 rotor; 24,000 rpm, 4°C). Fractions were tested for radioactivity.

Tube	Treatment		Radioactivity		
	Sonication time (secs)	Centrifugation time (hours)	Total (cps)	Pellet (%)	Fractions 4-13 inclusive (%)
X—X	1	60	13431	26.6	46.0
●—●	2	180	13680	29.4	38.9
O—O	3	180	12549	29.2	27.4

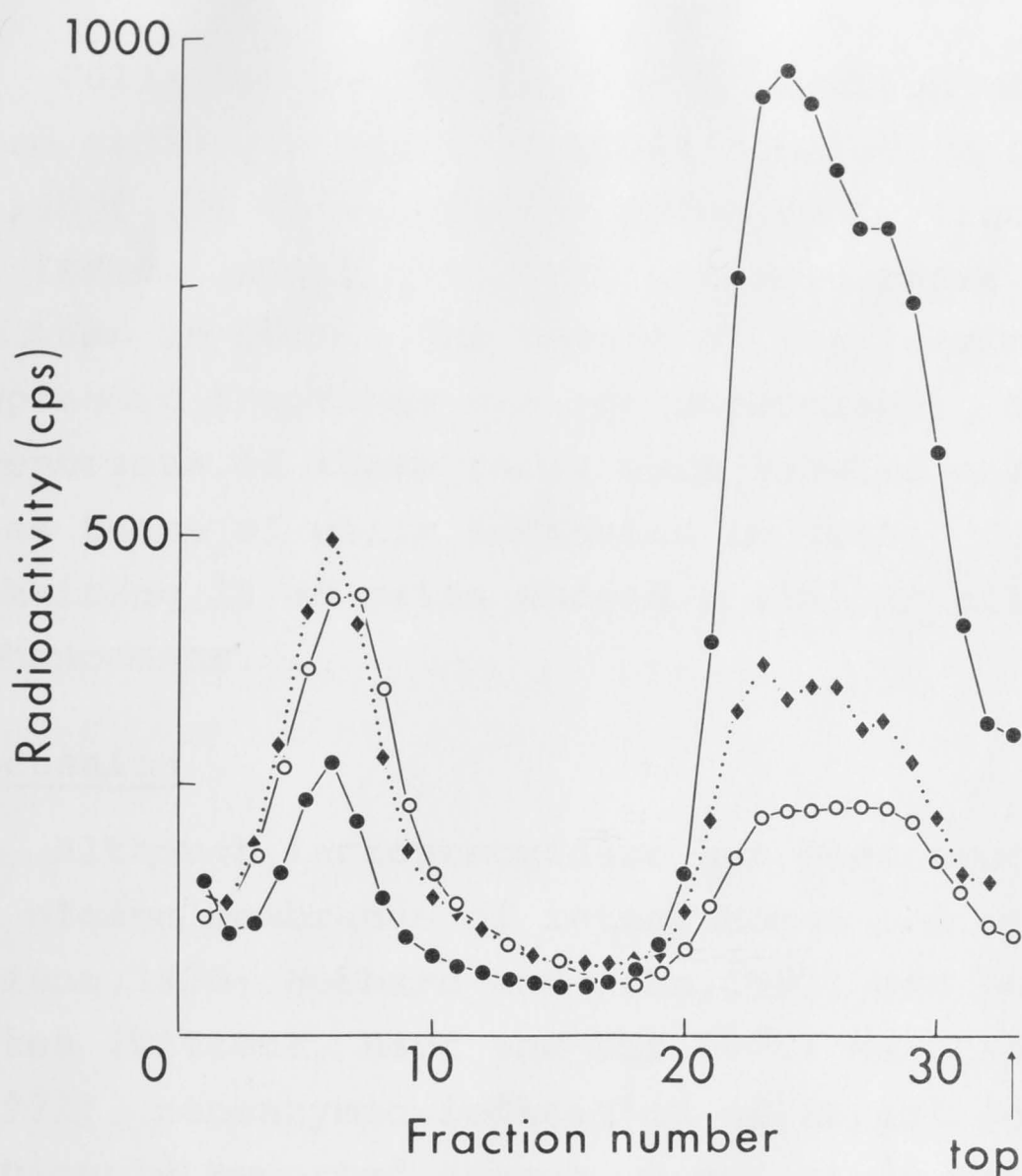


Figure 3.11: Effect of the buffer on CRBC iodination

CRBC (5×10^8 cells) in Alsever's solution were added to each of three tubes. The cells were washed 3 times with 10 ml aliquots of Alsever's solution (tube 1), DBSS (tube 2) or DBSS containing 15 mM NaN_3 (tube 3) before being suspended in a final volume of 200 μl of the respective buffer. Carrier-free Na^{125}I (1 μl , 6×10^{-5} M; 100 μCi) and chloramine-T (5 μl , 2×10^{-2} M) were added to each tube. After 5 min at 25°C , 5 μl 2×10^{-2} M $\text{Na}_2\text{S}_2\text{O}_5$ and then 5 ml of buffer were added. Each cell preparation was washed 5 times with 10 ml aliquots of buffer, suspended in 1 ml of buffer and diluted to 10 ml with 5 mM MgCl_2 /20 mM Tris-HCl, pH 7.4 before being sonicated (15 seconds) and centrifuged through a 20-50 % (w/v) sucrose gradient containing 5 mM MgCl_2 /20 mM Tris-HCl, pH 7.4 (SW25.1 rotor; 24,000 rpm, 14 h, 4°C). Fractions were collected and tested for radioactivity.

Solution used to label and wash CRBC	Radioactivity					
	Total cps	Pellet cps (%)	Plasma membrane (fns 2 - 12)		Cytoplasm (fns 20-31)	
			cps	(%)	cps	(%)
●—● Alsever's	14901	3383 (22.7)	1550	(10.4)	8404	(56.4)
○—○ DBSS	7703	1656 (21.5)	2789	(36.2)	2311	(30.0)
◆---◆ DBSS + azide	8997	1844 (20.5)	2771	(30.8)	3329	(37.0)

Cells labeled in Alsever's solution contained 1.93 times more radioactivity than those labeled in DBSS. The bulk of this label (56 %) was in the cytoplasmic fractions, suggesting that iodide influx into CRBC was more rapid in Alsever's solution than in DBSS. The nature of the labeled material in the cytoplasmic fractions was not determined. Moreover, the plasma membranes of these cells were labeled only 56 % as heavily as those of cells iodinated in DBSS. Cells labeled in DBSS containing 15 mM azide showed a similar although less marked phenomenon.

3.3 Discussion

Although lactoperoxidase has been successfully used to iodinate plasma membranes of intact human red cells (Phillips and Morrison, 1970; Hubbard and Cohn, 1972) and human and mouse lymphocytes (Vitteta, Baur and Uhr, 1971; Marchalonis, Cone and Santer, 1971), nonenzymic iodination of intact cells has not been previously reported except in one study which used labeled erythrocytes to correlate radioactivity with silver grains formed in autoradiographs (Ada, Humphrey, Askonas, McDevitt and Nossal, 1966). The work in this chapter indicates that erythrocyte plasma membranes can also be selectively labeled by iodination of intact cells using chloramine-T. A comparison of the ratio (specific activity of plasma membranes/specific activity of haemoglobin) obtained with human RBC using lactoperoxidase (2500/1; Hubbard and Cohn, 1972) with that obtained with SRBC in this study (560/1) shows there is little difference between the selectivity of the two methods.

Lactoperoxidase is claimed to be superior to chloramine-T for protein iodination as it can be used at lower oxidant (H_2O_2) concentrations (Marchalonis, 1969). Data concerning oxidation of proteins by H_2O_2 is not available however and there is little factual evidence to support such a claim. Moreover, iodination by lactoperoxidase requires incubation at 37°C for periods of 10-15 min. or longer (Marchalonis, 1969; Hubbard and Cohn, 1972), in contrast to chloramine-T iodination which is complete within several minutes at 0°C (Sonoda and Schlamowitz, 1970; this chapter). Any distinction must therefore compare the oxidation obtained

after long periods at 37°C using low concentrations of oxidant to that obtained after a shorter period at 0°C using higher concentrations. Whether such a distinction is necessary in any particular study clearly depends on whether or not the component(s) under investigation are altered by oxidation.

Using the chloramine-T method to label intact cells, SRBC membranes were iodinated 121 times more heavily than was the cytoplasm, on a protein wt/wt basis. Furthermore much of the cytoplasmic radioactivity was associated not with haemoglobin (the major cytoplasmic protein) but with traces of smaller proteins which on the basis of their specific radioactivities were almost certainly solubilized plasma membrane components. Washed ghosts had a specific activity of 924 cps/mg protein, compared to haemoglobin (1.7 cps/mg). The plasma membranes were therefore labeled 560 times more heavily than haemoglobin.

Approximately 30 % of radioactive material present in the cytoplasmic extracts of SRBC labeled for 5 min. at 25°C was of extremely low molecular weight and was probably free iodide, as a linear uptake of iodide by cells was observed during labeling. The rate of uptake at 0°C was 0.24 times that at 25°C, and as the iodination reaction (temperature-independent in this range) was complete within 1-2 min., the net uptake of iodide could if necessary be minimized to negligible levels without significantly affecting the extent of cell iodination.

Plasma membranes of both SRBC and CRBC could be isolated by equilibrium density centrifugation in good yield and purity as judged by recovery of radioactivity and protein. The membranes were identified by their high specific radioactivity and by their content of cell surface antigenic determinants. SRBC membranes were obtained in a narrow band (mean density, 1.142) in yields of at least 70 % based on recovery of label and surface antigens. CRBC plasma membranes were more difficult to isolate because of the more complex nature of these cells. Lysis by hypotonic shock was completely ineffective. This treatment released cytoplasmic components but did not disentangle the plasma membranes from nuclear material. Dounce homogenization in hypotonic solution released

some membranes, but sonication proved most effective and resulted in maximal yields after 30-45 seconds treatment. Longer periods fragmented the membranes so that prolonged centrifugation was required to sediment the pieces to their buoyant density, which was unchanged at 1.163 g/cc. This density is higher than reported by Zentgraf *et al.* (1971) (plasma membrane, 1.142 ± 0.024 ; nuclear membrane, 1.198 ± 0.037).

The finding that CRBC iodinated in Alsever's solution contained more intracellular label than did cells labeled in DBSS indicates the marked effect that different buffers can have on ion influx in cells. The presence in Alsever's solution, but not in DBSS, of glucose and citrate suggests that increased influx of iodide in Alsever's solution may have been the result of either increased metabolic activity (*i.e.* active iodide transport) or altered membrane permeability due to chelation of divalent ions by citrate. Calcium in particular is known to be important in membrane stabilization (Weiss, 1967; Rosenberg and Guidotti, 1969) and its removal from intact CRBC may increase permeability to ions such as iodide. However, a similar although less marked phenomenon was observed in cells labeled in DBSS containing azide and it is possible that neither of these mechanisms is involved. Iodination of cells in the presence of EDTA would determine the consequence of calcium depletion.

In conclusion, the chloramine-T method seems a satisfactory alternative to lactoperoxidase for selectively iodinating the plasma membranes of intact red blood (and possibly) other cells. In addition to the possible iodination of membrane proteins not accessible for iodination by lactoperoxidase, the chloramine-T method has the advantage of rapidity even at 0°C. This is particularly important for cellular work as it minimizes metabolic activity, especially membrane turnover, and also minimizes cellular uptake of iodide, which may create a high radioactive background and thus interfere with the detection of labeled proteins present only in minor quantities.

3.4 Summary

1. Methods for selectively labeling the plasma

membranes of intact cells have been evaluated. Radioiodine, which can be covalently linked to proteins by enzymic or chemical means, is superior in several ways to most other protein-labeling reagents.

2. Intact SRBC and CRBC were radioiodinated nonenzymically by chloramine-T oxidation. Iodination of SRBC was complete within 1-2 min. at either 0°C or 25°C. During prolonged incubation with iodide, a linear, temperature-dependent uptake was observed.

3. SRBC labeled for 5 min. at 25°C were fractionated. The plasma membrane ghosts contained 71 % of the cell-associated radioactivity and had a specific activity of 924 cps/mg protein. Approximately 55 % of the cytoplasmic radioactivity was associated with material concluded to be solubilized from the plasma membranes. Only 16 % of the cytoplasmic label was associated with haemoglobin (specific activity, 1.7 cps/mg) and on this basis the plasma membranes were labeled 560 times more heavily than was haemoglobin.

4. CRBC plasma membranes were also labeled selectively by iodination of intact cells. The plasma membranes contained at least 37 times more label than did the cytoplasm, compared to a similar figure of 121 for SRBC. As was also found with SRBC, only 31 % of the cytoplasmic label was precipitated in 10 % TCA. The proportion of label found intracellularly depended on the nature of the buffer used for cell iodination.

5. SRBC and CRBC plasma membranes were readily isolated by equilibrium density centrifugation on sucrose gradients. SRBC membranes (buoyant density, 1.14) were isolated in high yield by lysis of cells in hypotonic solution. CRBC plasma membranes (buoyant density, 1.16) were more difficult to isolate. Hypotonic shock and Dounce homogenization released little membrane material. Sonication was most effective and released the plasma membranes in good apparent yield from denser nuclear debris.

4.1 Introduction

4.2 Experimental results

4.3 Discussion

4.4 Summary

4.1 Introduction

When this work was begun, it was thought that the isolation and purification of lymphocyte plasma membranes was a logical first step in the characterization of the receptors for antigen. Thus the receptors might be labeled extrinsically by binding with antigens, with anti-Ig antibodies, or reacted with radioactive iodine. The plasma membrane could then be purified to remove cytoplasmic contamination and fractionated under various conditions to separate and characterize its components. Clearly, the success of an approach such as this depends on whether the membrane can be sufficiently purified without selective loss of some components.

Chapter 4

The isolation of plasma membranes from sheep lymphocytes easily and in what form is most obvious of these are (a) the characteristics of the tissue to be fractionated (e.g. solid organ versus free-floating cells; cell size, shape, rigidity and heterogeneity), (b) the selection of adequate markers, and (c) the methods chosen for tissue and cell disruption, fractionation and analysis of components (Warren, Glick and Kass, 1966; Hoyle, 1967; De Pierre and Karnovsky, 1973). It is characteristic of many studies that several methods are initially applied to a single tissue and the one proving most successful is then adopted for routine use.

Although plasma membranes have been isolated from a variety of mammalian cells (e.g. liver: Evans, 1971; Hanning, Kaulen and Stoffel, 1976; cells: Borzan, Hagopian and Elyar, 1968; L-cell: Erdemite and Till, 1971; leucocytes: Woodin and Wisniewski, 1966), there is only one detailed description of their isolation from lymphocytes (Allan and Crumpton, 1970). The purified lymphocyte membrane has been dissolved in detergents and its components fractionated and partially characterized (Allan and Crumpton, 1971). Although these authors have also investigated the properties of the receptors (present on the surface of pig lymphocytes) for the mitogen phytohemagglutinin

- 4.1 Introduction
- 4.2 Experimental results
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4.1 Introduction

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A number of factors are important in determining how easily and in what form plasma membranes can be isolated. The most obvious of these are (a) the characteristics of the tissue to be fractionated (e.g. solid organ versus free-floating cells; cell size, shape, rigidity and heterogeneity), (b) the selection of adequate markers, and (c) the methods chosen for tissue and cell disruption, fractionation and analysis of components (Warren, Glick and Nass, 1966; Boyle, 1967; De Pierre and Karnovsky, 1973). It is characteristic of many studies that several methods are initially applied to a single tissue and the one proving most successful is then adopted for routine use.

Although plasma membranes have been isolated from a variety of mamalian cells (e.g. *liver*: Evans, 1971; Henning, Kaulen and Stoffel, 1970; *HeLa cells*: Bosman, Hagopian and Eylar, 1968; *L-Cells*: Brunette and Till, 1971; *leucocytes*: Woodin and Wieneke, 1966), there is only one detailed description of their isolation from lymphocytes (Allan and Crumpton, 1970). The purified lymphocyte membrane has been dissolved in detergent and its components fractionated and partially characterized (Allan and Crumpton, 1971). Although these authors have also investigated the properties of the receptors (present on the surface of pig lymphocytes) for the mitogen phytohaemagglutinin

(Allan, Auger and Crumpton, 1971; Allan and Crumpton, 1973), they have not as yet reported studies on the receptors for antigen.

This chapter describes experiments designed to establish a standard procedure for the isolation of "pure" plasma membranes from free-floating sheep lymphocytes.

4.2 Experimental results

4.2.1 Disruption of sheep lymphocytes

Dounce homogenization, although a gentler and therefore perhaps more preferable method of cell disruption than ultrasonic rupture, did not cause significant morphological damage to sheep lymphocytes as judged by phase-contrast microscopy. As such homogenization was also relatively ineffective in releasing plasma membrane material from lysed CRBC (Chapter 3), the time required to sonically-disrupt lymphocytes was determined.

Lymphocytes, suspended in either isotonic (150 mM Cl^-) or hypotonic (25 mM Cl^-) solution, were sonicated for 0, 5, 30, or 60 seconds and then examined visually for gross structural damage (phase-contrast) and for their ability to exclude trypan blue. The results are shown in Table 4.1.

Suspension in hypotonic buffer caused 82 % of the cells to become permeable to trypan blue although none appeared significantly swollen. Such cells were more susceptible to sonic fragmentation than were cells in isotonic solution. However, at least 30 seconds sonication of either suspension was required to fragment ≥ 80 % of the cells. Sonication for 60 seconds reduced in both cases the number of discernible cells or nuclei to less than 1 % of those originally present.

4.2.2 Fractionation of sonicated ^{125}I -labeled sheep lymphocytes

To determine whether the plasma membranes of ^{125}I -labeled sheep lymphocytes could be readily identified by density gradient centrifugation like those of RBC (Chapter 3), lymphocytes were labeled with ^{125}I using the chloramine-T method, washed and sonicated before being layered over sucrose gradients and subjected to centrifugation (Fig 4.1).

The resulting radioactivity profile was the same for

Table 4.1

Ultrasonic disruption of sheep lymphocytes

Buffer in which lymphocytes were sonicated	Period of sonication (seconds) ^{a)}	Number of cells which excluded trypan blue	Number of 'intact' cells or nuclei stained with trypan blue	Total number of intact cells or nuclei (% of original)	% of remaining cells which excluded trypan blue	Description (visual appearance)
<u>ISOTONIC</u> (DBSS)	0	406	24	430 (100)	95	All small, intact cells
	5	255	115	370 (86)	69	Little debris
	30	27	65	92 (21)	29	Mostly debris
	60	0	6	6 (1)	0	" "
<u>HYPOTONIC</u> (5 mM MgCl ₂ , 20 mM Tris-HCl, pH 7.5)	0	83	380	463 (100)	18	Small, intact, stained cells
	5	8	160	168 (36)	5	Mostly fragmented cells
	30	0	37	37 (8)	0	Mostly cell debris
	60	0	5	5 (1)	0	" " "

a) Lymphocytes were suspended at 2×10^7 cells/ml in DBSS or Tris buffer and 10 ml aliquots were sonicated at 0°C for the indicated periods. To determine the extent of cell damage, each sample was (a) viewed under phase-contrast microscopy and (b) incubated with a 0.5 % (w/v) trypan blue solution.

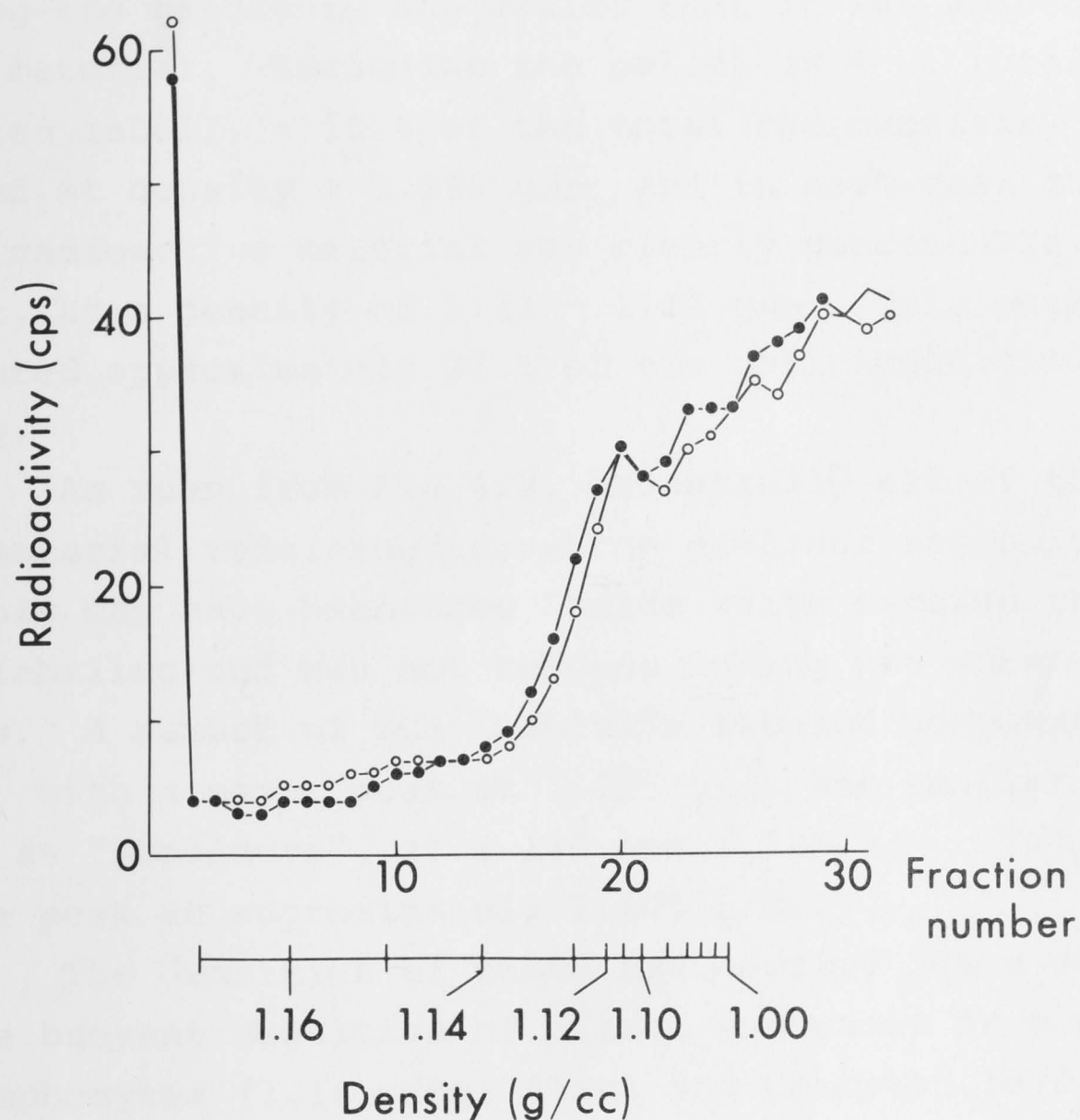


Figure 4.1: Radioactivity profile obtained after gradient centrifugation of sonicated ^{125}I -labeled sheep lymphocytes

Lymphocytes (1×10^8 cells in 0.2 ml of DBSS) were labeled with ^{125}I (200 μCi) by reaction for 3 min at room temperature in the presence of $5 \times 10^{-4}\text{M}$ chloramine-T. The cells were washed at $0-4^\circ\text{C}$ in DBSS/10 % NRS/15 mM NaN_3 , divided into 2 equal portions and sedimented by centrifugation. One cell pellet was resuspended in 10 ml of 5 mM MgCl_2 /20 mM Tris-HCl, pH 7.5, the other in 10 ml of 5 mM MgCl_2 /130 mM NaCl/20 mM Tris-HCl, pH 7.5. Each suspension was sonicated for 60 seconds and then centrifuged on a 30-45 % (w/v) sucrose gradient of similar ionic composition (SW25.1 rotor; 21,000 rpm, 11 h, 5°C). Fractions were collected and assayed for radioactivity.

●—●—● Isotonic
 ○—○—○ Hypotonic

cells sonicated and centrifuged in either isotonic or hypotonic Tris-buffer. Much of the radioactivity remained in the region overlying the gradient, suggesting that it was associated with soluble material. Excluding the pellet (8 % of total cell-associated label), < 10 % of the total radioactivity was recovered at density > 1.135 g/cc, and in each case only one peak of radioactive material was clearly discernable in the gradient, at a density of 1.11 - 1.12 g/cc. This peak constituted approximately 25 % of the cell-associated radioactivity.

As seen from Fig 4.2, essentially all of the radioactive material remaining above the gradient was soluble in TCA. This may have been free iodide which entered the cells during labeling and was not removed during the subsequent washings. A number of TCA-insoluble labeled components were evident, with a major peak at 1.105 g/cc, and smaller peaks, evident as "shoulders", at 1.115 and 1.125 g/cc. The A_{280} also showed a peak at approximately 1.105 g/cc.

The densities of these radioactive peaks were lower than the buoyant densities of plasma membranes from pig lymph node lymphocytes (1.14 g/cc; Allan and Crumpton, 1970) and rabbit polymorphonuclear leucocytes (1.15 g/cc; Woodin and Wieneke, 1966). This difference might have occurred if (a) sheep lymphocyte plasma membranes are less dense than those of other cell types, including lymphocytes from other animals, (b) the ^{125}I -labeled membrane components become separated partially or completely from the membrane during sonic cell disruption, or (c) intracellular (as well as plasma membrane) components are labeled by iodination of sheep lymphocytes.

To distinguish between these possibilities it was important to determine firstly, the buoyant density of the *intact* plasma membrane and secondly, whether during its isolation the membrane is broken into fragments of dissimilar composition. Thus a reliable plasma membrane marker was required. ^{125}I -Labeled anti-sheep-lymphocyte globulin (ALG) seemed suitable for this purpose, for the following reasons: (a) The antibody bound to all sheep lymph cells as determined by autoradiography, whereas normal rabbit IgG did not bind in detectable amounts. Furthermore, this ALG did not bind to mouse lymphocytes, thus showing

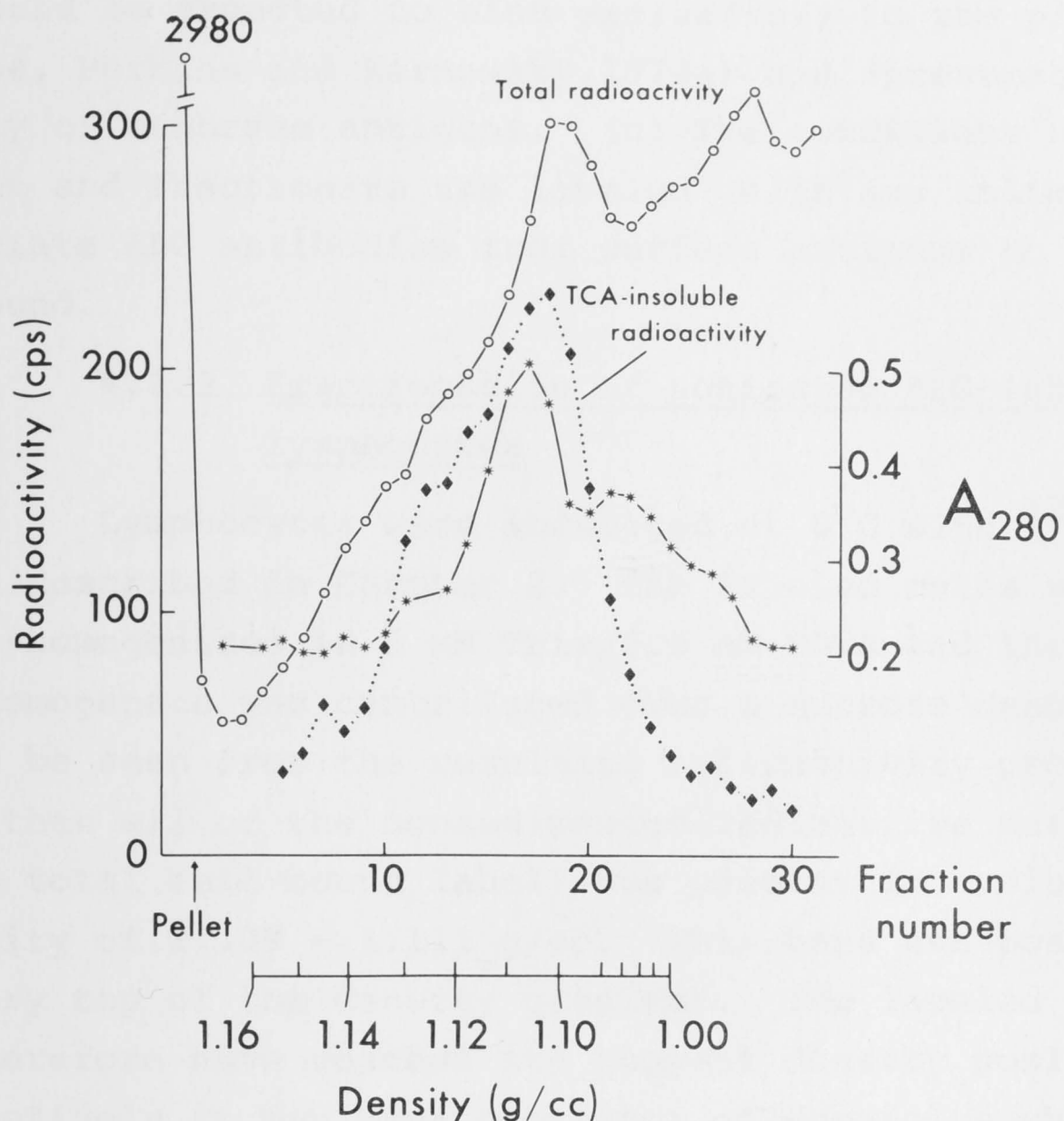


Figure 4.2: Radioactivity profile obtained after gradient centrifugation of sonicated ^{125}I -labeled sheep lymphocytes. Precipitation with trichloroacetic acid

Lymphocytes were labeled with ^{125}I by a procedure similar to that described in Fig. 4.1. The labeled cells were washed and then sonicated for 60 seconds after suspension in 10 ml of 5 mM MgCl_2 /130 mM NaCl /20 mM Tris-HCl , pH 7.5. The sonicate was centrifuged over a 25-45 % (w/v) sucrose gradient (SW25.1 rotor; 21,000 rpm, 6 h, 5°C) and 1 ml fractions were collected. Each fraction was tested for total radioactivity and A_{280} .

For measurement of TCA-insoluble radioactivity, 1 ml of 1 N TCA was added to each fraction and after 15 min at 0°C , the samples were centrifuged at 1000g/30 min/ 4°C . The supernatants were then removed and the radioactivity in each sediment was determined.

its specificity. (b) Under the conditions used to label intact lymphocytes (0°C in the presence of 15 mM NaN_3), ^{125}I -labeled ALG could be expected to bind *exclusively* to the plasma membrane (Unanue, Perkins and Karnovsky, 1972a) and (presumably) to a variety of membrane antigens. (c) The conditions used to disrupt and fractionate the labeled cells are unlikely to dissociate ALG antibodies from surface antigens to which they are bound.

4.2.3 Fractionation of sonicated ALG-labeled lymphocytes

Lymphocytes were incubated at 0°C with ^{125}I -labeled ALG as described in Chapter 2. The labeled cells were washed, Dounce-homogenized in 5 mM Tris/0.2 mM EDTA and the unfractionated homogenate was centrifuged over a sucrose density gradient. It can be seen from the resulting radioactivity profile (Fig 4.3a) that all of the nonsedimented radioactive material (72 % of the total cell-bound label) was present in a single band at a density of 1.109 - 1.111 g/cc. This band was positioned at the very top of the density gradient. The labeled material may therefore have reached its buoyant density position or alternatively it may have consisted of particles which were too small to sediment to their buoyant density during the period of centrifugation (12 h at 60,000 g_{av}).

Did the ALG dissociate from the antigens on the plasma membrane? To answer this question, material in the peak fraction of Fig 4.3a was analyzed by sedimentation centrifugation. Nearly all of the labeled material was larger than the free 7S antibody (Fig 4.3b). It was therefore considered that the antibodies had not dissociated from the membrane antigens to which they had initially bound.

Were (ALG/membrane antigen) complexes released from the plasma membrane by disintegration of the latter? This seemed a distinct possibility. Divalent cations, especially Ca^{++} and Mg^{++} , are known to be important in the stabilization of membrane structure (Williams, 1970; Weiss, 1967), and the EDTA present in the homogenizing buffer may have removed such cations and caused the membrane to disintegrate. It was considered that the net ionic strength of the medium may also be important in this respect.

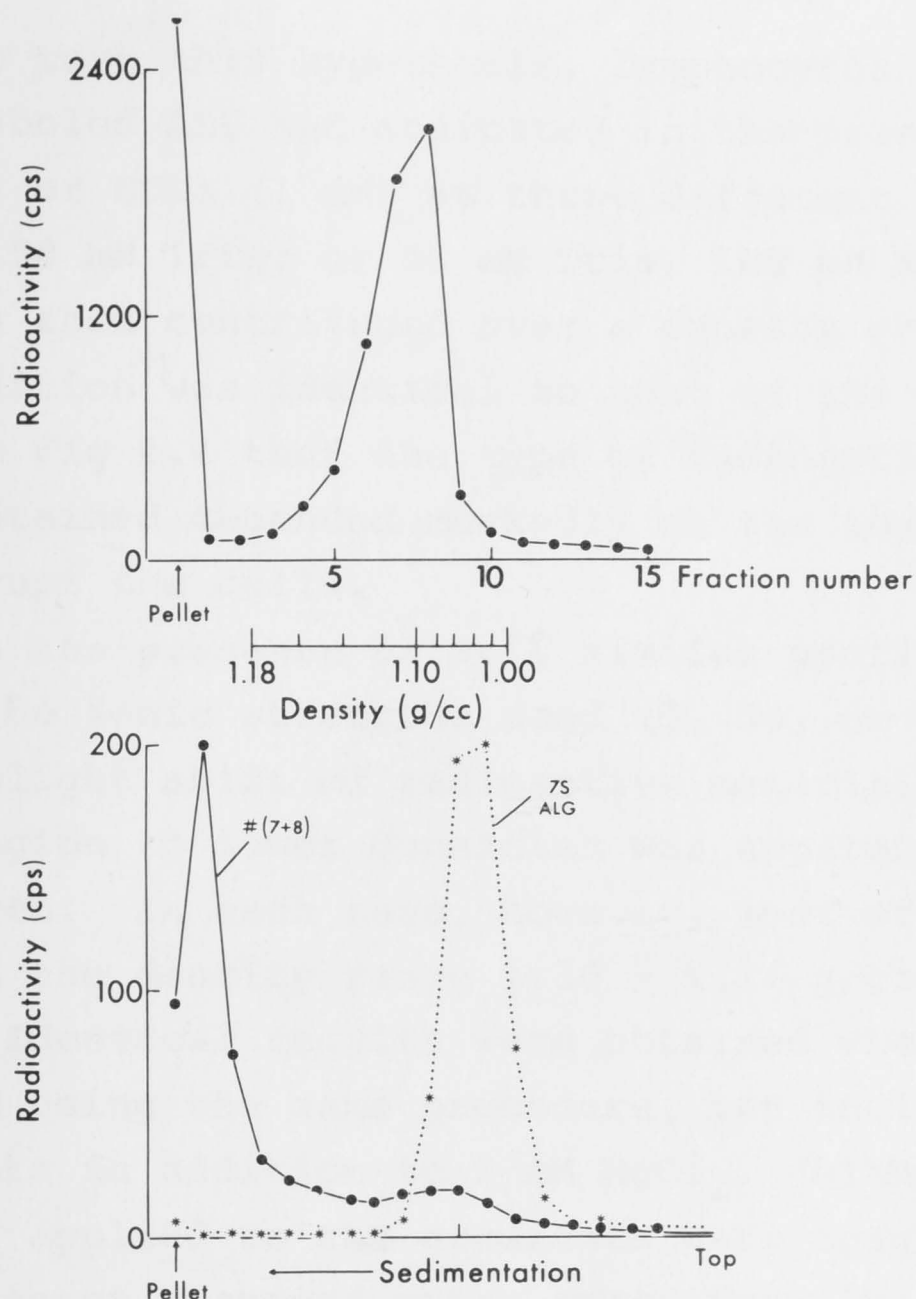


Figure 4.3:

TOP: Radioactivity profile resulting from gradient centrifugation of Dounce homogenized ^{125}I -ALG-labeled sheep lymphocytes

Lymphocytes (1×10^8 cells in 1 ml of DRN*) were incubated for 4 h at 0°C with $5 \mu\text{g}$ of ^{125}I -labeled ALG, washed well with DRN and then suspended in 7 ml of 0.2 mM EDTA/5 mM Tris-HCl, pH 7.5 at 0°C . The suspension was Dounce homogenized (20 strokes) and then centrifuged on a 25-50 % (w/v) sucrose gradient of similar ionic composition (SW25.3 rotor; 20,000 rpm, 12 h, 5°C). Fractions (1 ml) were assayed for radioactivity.

BOTTOM: Sedimentation profile of 7S ALG and labeled material in fractions (7,8) from above

Fractions 7 and 8 from the above experiment were pooled, dialysed against 0.2 mM EDTA/5 mM Tris-HCl, pH 7.5 and then sedimented through a 5-25 % sucrose gradient (SW65 rotor; 60,000 rpm, 11 h, 5°C). A sample of unreacted 7S ALG was similarly centrifuged.

*DRN: DBSS containing 10 % normal rabbit serum and 15 mM NaN_3 .

To test this hypothesis, lymphocytes were labeled with ^{125}I -labeled ALG and sonicated in the presence of either MgCl_2 (5 mM) or EDTA (1 mM) at three different ionic strengths (5 mM Tris; 50 mM Tris; or 50 mM Tris, 100 mM NaCl). Each sonicate was then centrifuged over a density gradient whose ionic composition was identical to that of the sample. It can be seen from Fig 4.4 that the type of radioactivity profile which was obtained depended markedly on the ionic conditions used to disrupt the cells.

In the presence of Mg^{++} , similar profiles were obtained at each of the ionic strengths used (5, 50, or 150 mM Cl^-), although a slight shift of radioactive material from the 1.14 - 1.18 g/cc region to lower densities was apparent with decreasing ionic strength. In each case, however, most of the label was recovered in the density range 1.10 - 1.14 g/cc, with a peak at 1.12 g/cc. Identical results were obtained when the experiment was repeated using the same procedure, but including CaCl_2 (2 mM) in the buffers in addition to 5 mM MgCl_2 . Although the preparations applied to the gradients were opaque, a band of opacity was never observed after centrifugation.

In the presence of EDTA, the type of radioactive profile obtained in the density gradients was affected significantly by changes in the net ionic strength. Using isotonic conditions (150 mM Cl^-) the profile was almost identical to that obtained in the presence of Mg^{++} . However an increasing proportion of the labeled material was recovered at 1.11 - 1.13 g/cc as the ionic strength was decreased and using 5 mM Tris, this density range contained 70 % of the total radioactivity. This effect was not due to dissociation of the ALG from membrane antigens, as labeled material recovered in the 1.11 - 1.12 g/cc range (using 5 mM Tris and either Mg^{++} or EDTA) was considerably larger than the free antibody as determined by sedimentation centrifugation (*inset*, Fig 4.4). Moreover, the effect was not restricted to ALG-labeled material, as large amounts of protein were also solubilized at low ionic strength by EDTA (Fig 4.5).

At low ionic strength in the presence of EDTA, the cell extract was essentially clear, whereas at higher ionic strengths, although the cell extracts were opaque, no band of opacity was observed after centrifugation.

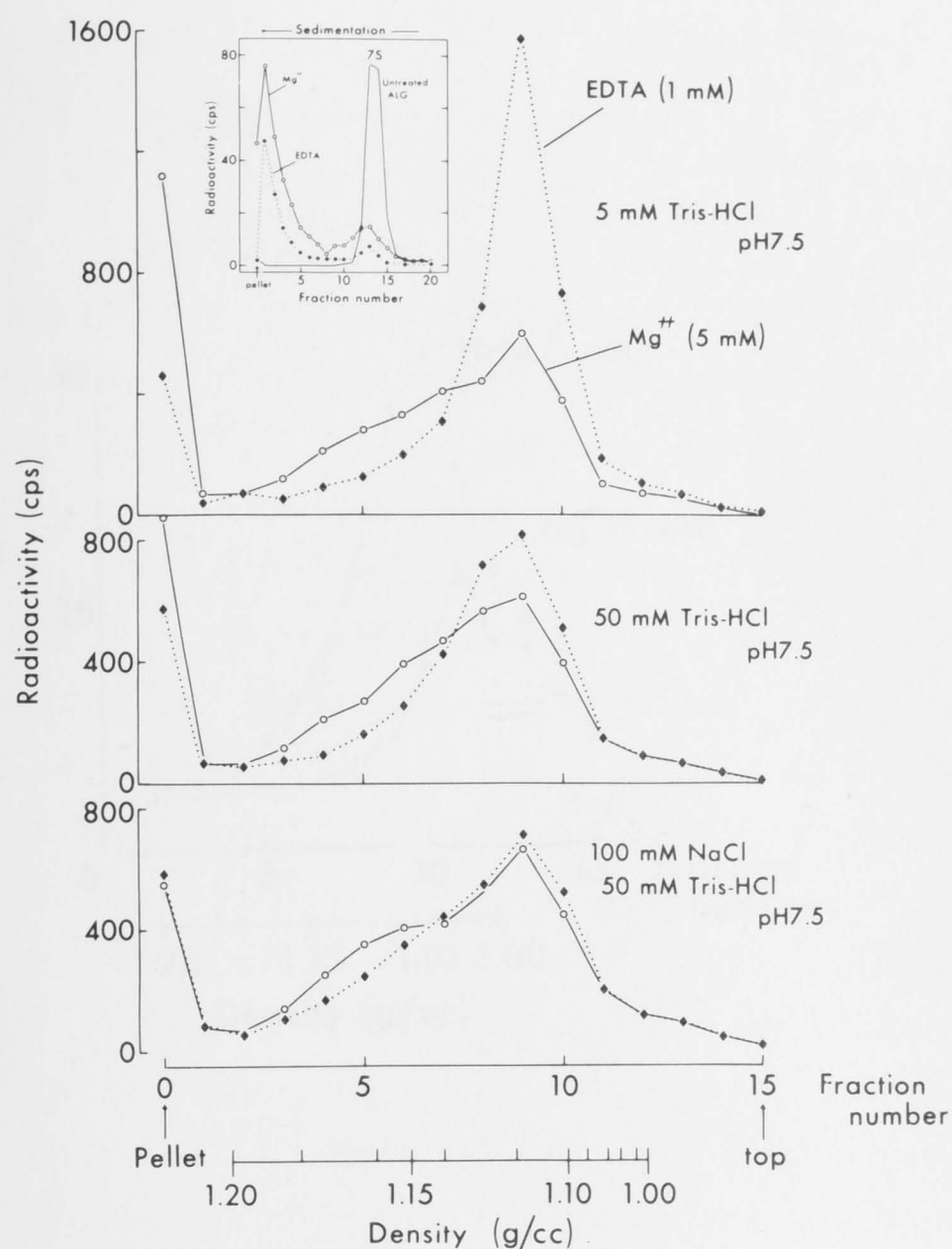


Figure 4.4: Radioactivity profiles obtained in density gradients after sonic rupture and gradient centrifugation of ^{125}I -ALG-labeled sheep lymphocytes in buffers of different ionic composition

Sheep lymphocytes, incubated in DRN* with ^{125}I -labeled ALG ($1\text{ }\mu\text{g ALG}/1 \times 10^8\text{ cells/ml}$, 2 h, 0°C) and washed several times at $0-4^\circ\text{C}$ in DRN, were rinsed once in $5\text{ mM MgCl}_2/100\text{ mM NaCl}/50\text{ mM Tris-HCl}$, pH 7.5 and divided into 6 equal fractions of 1×10^8 cells each. The suspensions were centrifuged and each cell pellet was then resuspended in 5 ml of one of six different Tris buffers (as indicated in the figure) before being sonicated (60 seconds) and centrifuged (SW25.3 rotor; 22,000 rpm, 12 h, 5°C) on a 25-50 % (w/v) sucrose density gradient containing the same buffer that was used to suspend the cells for sonication. Fractions (1 ml) were collected from each gradient and assayed for radioactivity.

*DRN: DBSS containing 10 % normal rabbit serum and 15 mM NaN_3 .

The inset indicates the radioactivity profiles which were obtained after sedimentation centrifugation of (a) untreated 7S ALG or (b) the pooled fractions (8,9 and 10) from the 5 mM Tris-EDTA or $-\text{Mg}^{++}$ gradients. The fractions were dialysed against their respective buffers before being centrifuged over 5-25 % sucrose gradients (SW65; 60,000 rpm, 6 h, 5°C).

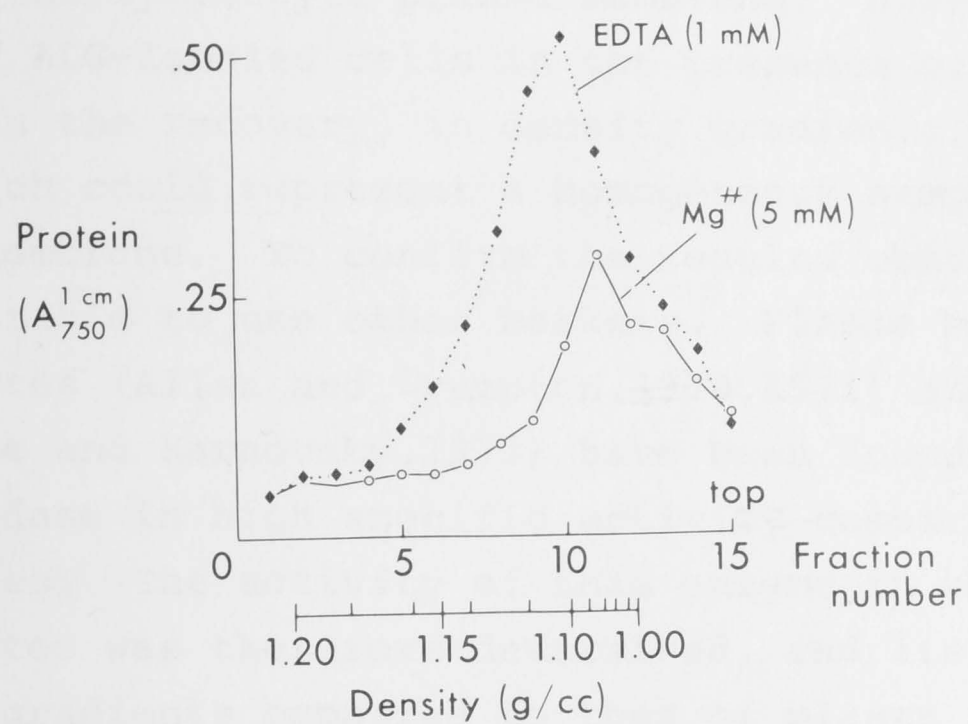


Figure 4.5: Distribution of proteins in density gradients after sonication and gradient centrifugation of sheep lymphocytes in 5 mM Tris buffer containing either EDTA or MgCl₂

Fractions from the 5 mM Tris gradients of Fig. 4.4 were tested for their protein content by reaction with Folin's reagent. Relative protein concentration is indicated by A₈₂₀.

4.2.4 The distribution of sheep lymphocyte 5'-nucleotidase in density gradients

The results presented above show clearly that certain ionic conditions, particularly those in which divalent cations are present, are important in maintaining the structural integrity of the lymphocyte plasma membrane. However the fractionation of ALG-labeled cells in the presence of Mg^{++} did not result in the recovery, in density gradients, of any distinct band which could represent a homogeneous sample of the original plasma membrane. To confirm the results obtained using ALG, it was desirable to use other markers. Plasma membranes of pig lymphocytes (Allan and Crumpton, 1970, 1971) and other cells (see De Pierre and Karnovsky, 1973) have been found to contain 5'-nucleotidase in high specific activity compared to other cellular structures. The activity of this enzyme in sonicates of sheep lymphocytes was therefore determined, and its distribution in density gradients compared to that of plasma membrane-bound ALG.

Enzyme activity was measured by the release of inorganic phosphate (Pi) from adenosine 5'-monophosphate during a 15 min. incubation at 37°C. Pi, measured as described in Chapter 2, was detected by A_{820} . It can be seen from Fig 4.6 that in the range 0-1.6, A_{820} was linearly dependent on Pi concentration. Furthermore, the total 5'-nucleotidase activity of cell extracts was directly proportional to the number of (sonicated) cells used in the assay. Extracts kept at 4°C for up to 21 h showed no loss in activity, but possessed 30 % more activity than did freshly-sonicated cells. Sucrose (25 % w/v) had no effect on the activity of the enzyme.

Fig 4.7 shows the distribution of sheep lymphocyte 5'-nucleotidase activity after the cells had been Dounce homogenized or sonicated in hypotonic Tris- Mg^{++} buffer and centrifuged over sucrose density gradients. In each case the total recovered activity was approximately 70 % of that applied to the gradients. Most of the activity was recovered near the top of the gradient, between density 1.09 and 1.12 g/cc, although activity was present in decreasing amounts at higher densities. The distribution of 5'-nucleotidase activity was therefore similar but not identical to that of membrane-bound ALG.

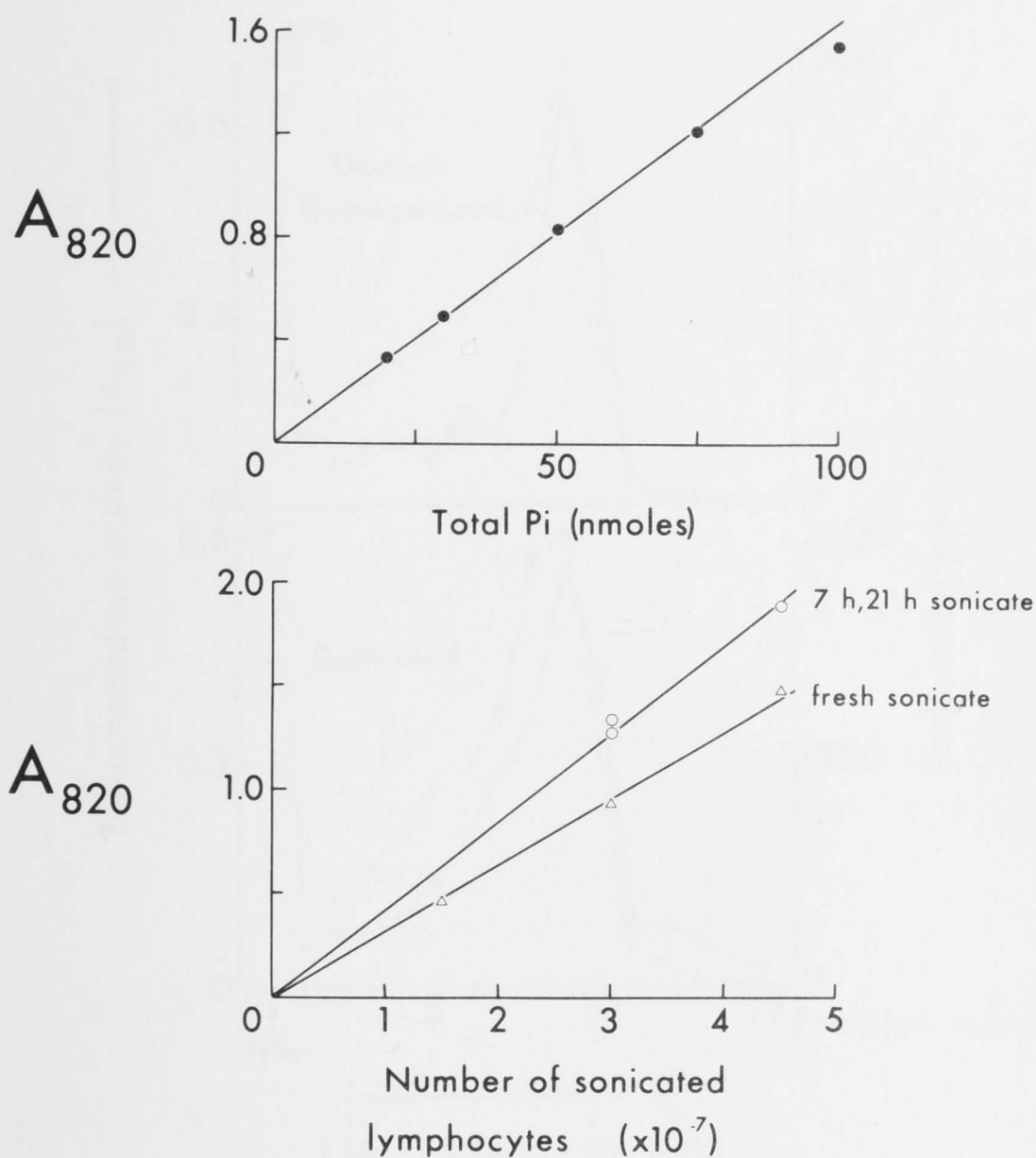


Figure 4.6:

TOP: Standard curve depicting the relationship between A_{820} and inorganic phosphate (Pi) for Pi determination

Known amounts of inorganic phosphate were reacted with molybdate/ascorbic acid, according to Ames and Dubin (1960).

BOTTOM: Standard curve showing the relationship between observed 5'-nucleotidase activity (A_{820}) and the number of sonicated sheep lymphocytes used in the assay system.

Lymphocytes (1×10^8 cells/ml in 5 mM $MgCl_2$ /100 mM NaCl/50 mM Tris-HCl, pH 7.5) were sonicated for 60 seconds. Aliquots were then assayed for 5'-nucleotidase activity as described in Chapter 2. Samples were assayed immediately (fresh sonicate) or 7 h or 21 h after sonication. The sonicates were incubated at $0^\circ C$ prior to assay.

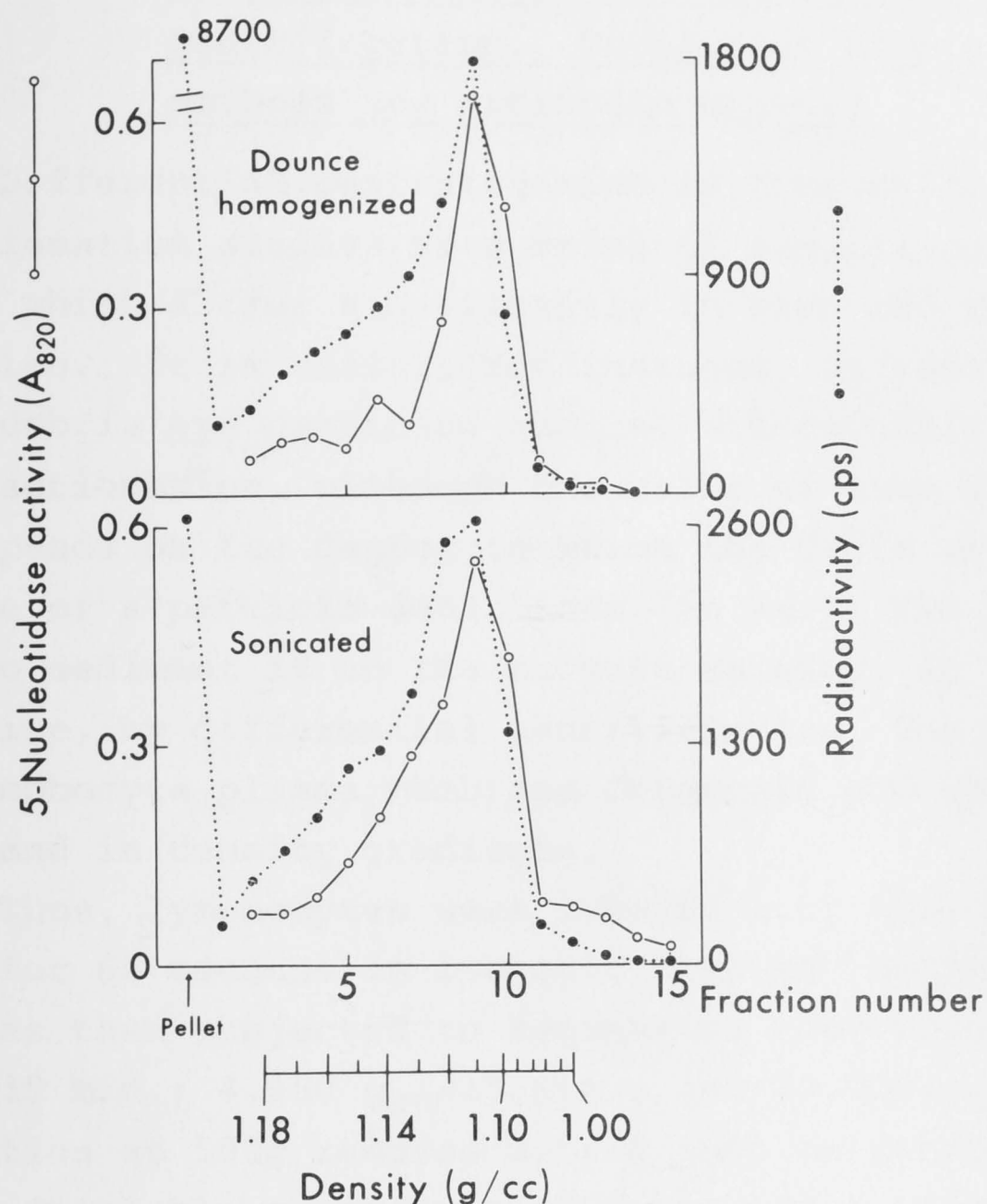


Figure 4.7: Distribution in density gradients of 5'-nucleotidase activity and radioactivity after gradient centrifugation of Dounce homogenized or sonicated ^{125}I -ALG-labeled sheep lymphocytes

Lymphocytes, labeled for 2 h at 0°C with ^{125}I -labeled ALG ($5\text{ }\mu\text{g}$ ALG/ 1×10^8 cells/ml, in DRN*), were washed and divided into equal portions of 5×10^8 cells each. After sedimentation, each cell pellet was suspended in 5 ml of 5 mM MgCl_2 /5 mM Tris-HCl, pH 7.5. One suspension was Dounce homogenized (30 strokes), the other sonicated for 60 seconds. Each suspension was then centrifuged over a 25–50 % (w/v) sucrose gradient (SW25.3 rotor; 22,000 rpm, 18 h, 5°C). Fractions (1 ml) were collected from each gradient and assayed for radioactivity and also for 5'-nucleotidase activity.

*DRN: DBSS containing normal rabbit serum and 15 mM NaN_3 .

4.2.5 Fractionation of sheep lymphocytes by differential centrifugation and density gradient centrifugation. Comparison of disruption methods and extrinsic markers

Differential centrifugation is frequently used in cell fractionation studies as a means of separating cellular components which differ significantly in size and rate of sedimentation. It is useful, for instance, in removing insoluble debris and particles such as mitochondria prior to further fractionation, although the value of this procedure clearly depends on the degree to which the cells are disrupted. As the size of a particle determines (in part) the time required to sediment it to its buoyant density, it was decided to determine, by differential centrifugation, the relative size of lymphocyte plasma membrane fragments and where each of these band in density gradients.

Thus, lymphocytes were labeled with ALG, washed and sonicated for 60 seconds in isotonic Tris-Mg⁺⁺ buffer. The sonicate was then subjected to sequential centrifugation steps of 500g_{av}/15 min.; 4,000 g_{av}/15 min.; and 20,000 g_{av}/30 min. Centrifugation at 500g removes debris such as unbroken cells, nuclei and insoluble aggregates, whereas the 4,000g step sediments smaller structures (e.g. mitochondria; Allan and Crumpton, 1970). After resuspending the 4,000g and 20,000g pellets in buffer, these, and an aliquot of each supernatant were each centrifuged over a 25-60 % (w/v) sucrose gradient. The resulting radioactivity profiles are shown in Fig 4.8.

The 500g step eliminated labeled material which sedimented completely through the gradient. Except for this difference, however, the profiles obtained with the unfractionated sonicate and the 500g supernatant were identical. The 4,000g spin also removed very little labeled material (< 10 % of total), and the supernatant from this step contained 87 % of the total cell-associated radioactivity, and had a density profile very similar to that of the crude sonicate.

Centrifugation at 20,000g for 30 min. sedimented 49 % of the labeled material present in the 4,000g supernatant. This treatment should have sedimented all particles having a sedimentation co-efficient of > 400S, and therefore material remain-

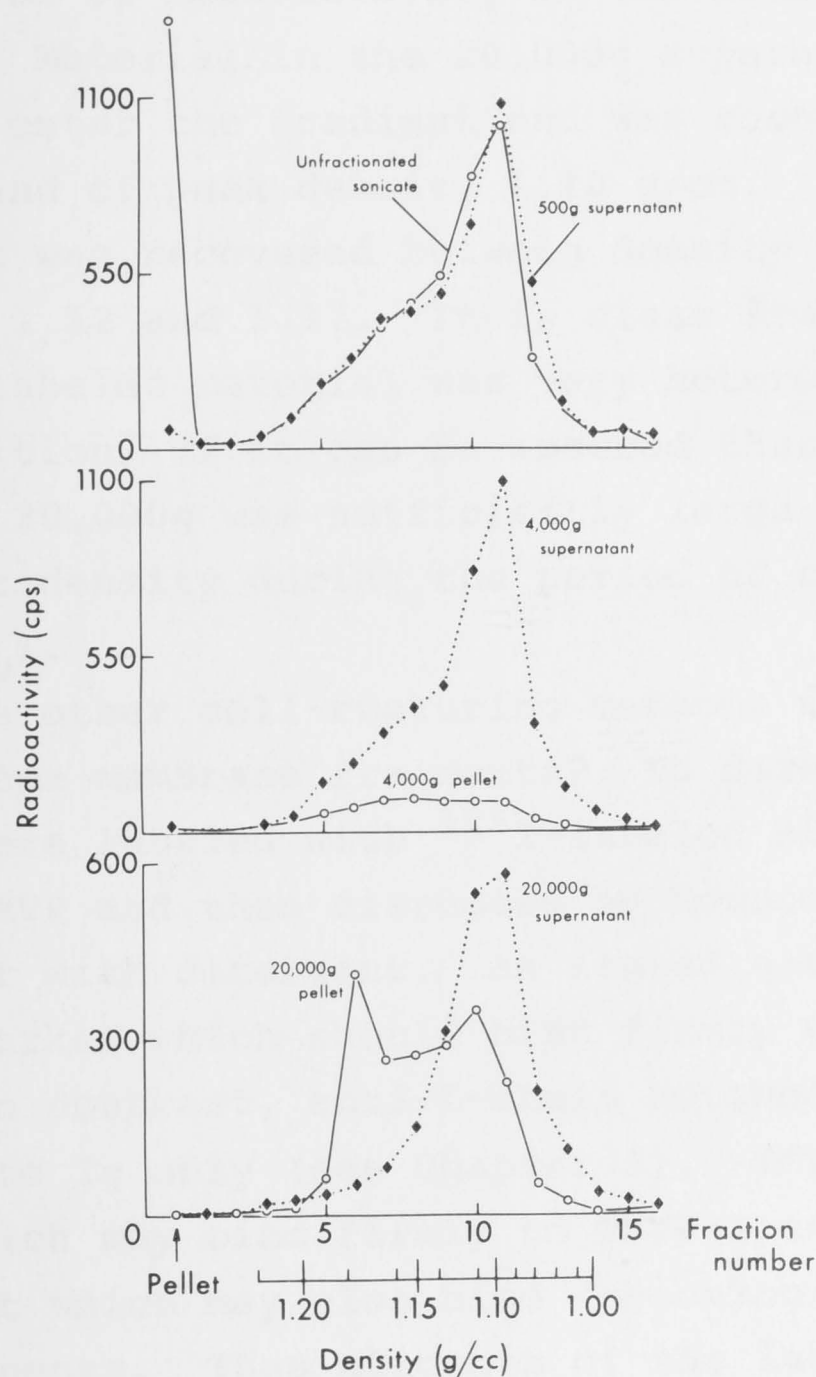


Figure 4.8: Radioactivity profiles resulting from density gradient centrifugation of different fractions obtained by differential centrifugation of sonically-disrupted ^{125}I -ALG-labeled sheep lymphocytes

Lymphocytes were incubated at 0°C with ^{125}I -labeled ALG ($1\text{ }\mu\text{g ALG}/1 \times 10^8$ cells/ml of DRN*; 90 min) and washed well in DRN before being suspended at 1×10^8 cells/ml in $5\text{ mM MgCl}_2/100\text{ mM NaCl}/50\text{ mM Tris-HCl}$, pH 7.5 and sonicated for 60 seconds. Aliquots of the sonicate were then fractionated by sequential centrifugation steps of $500\text{g}/15\text{ min}$; $4,000\text{g}/15\text{ min}$; and $20,000\text{g}/30\text{ min}$. The sediment resulting from each step was resuspended in buffer, and aliquots of each sediment or supernatant were then centrifuged over 25-60 % (w/v) sucrose density gradients (SW25.3 rotor; 22,000 rpm, 18 h, 5°C). Fractions (1 ml) were collected from each gradient and assayed for radioactivity.

*DRN: DBSS containing 10 % normal rabbit serum and 15 mM NaN_3 .

ing in the supernatant may be regarded as highly fragmented, and possibly soluble. This conclusion is substantiated by the distribution of radioactivity in the density gradient (Fig 4.8, bottom). Material in the 20,000g supernatant did not significantly enter the gradient and was recovered in a nearly symmetrical band of peak density 1.10 g/cc. In contrast, the 20,000g pellet was recovered between density 1.10 and 1.18 g/cc, with peaks at 1.12 and 1.17. It is clear from these results that the ALG-labeled material was very heterogeneous in size and in composition, if it can be assumed that all the material sedimented at 20,000g was sufficiently large to be sedimented to its buoyant density during the period of centrifugation (18 h at 61,000g_{av}).

Were other cell-rupturing methods more suitable for preparing plasma membrane fragments? To determine this, lymphocytes were labeled with ¹²⁵I-labeled ALG, anti-L-chain antibody or TMVP and then disrupted by Dounce homogenization, sonication, or with detergent. As stated earlier, ALG was chosen as a marker which should bind firmly to surface antigen(s) except Ig. In contrast, anti-L-chain antibodies bind specifically to Ig only (see Chapter 5). TMVP was selected as an antigen which may bind firmly to TMVP-specific antigen-receptors, but which may also bind (by unknown means) to other surface components. Thus aliquots of the labeled cells were disrupted by each of the methods mentioned above and fractionated by centrifugation at 500g, 4,000g, and 20,000g. The results are given in Table 4.2.

It is evident that Nonidet P-40 (NP40) solubilized > 71 % of the radioactivity from cells labeled with any one of the three protein markers and in each case, almost all of the NP40-solubilized label was too small to be sedimented by 30 min. centrifugation at 20,000g. Sonication caused a similar effect, fragmenting > 80 % of each cell-associated marker to a form not sedimented by centrifugation at 4,000g. However, a large fraction of this material was sedimented at 20,000g (ALG, 28 %; anti-L, 35 %; TMVP, 47 %). This suggests that the labeled material present in NP40 cell extracts is smaller in size than that in cell sonicates. Less than 30 % of the label in NP40 extracts of ALG- or anti-L-chain labeled cells was present in dissociated (7S) form as determined by sedimentation

Table 4.2

Fractionation by differential centrifugation of sheep lymphocytes after incubation with ^{125}I -labeled ALG, anti-L-chain antibody or TMVP and disruption by Dounce homogenization, sonication or detergent treatment

		Centrifugation step					
125 I-Labeled protein used to label lymphocytes ^{a)}	Method used to fragment labeled cells ^{b)}	500xg/15 min.		4,000xg/15 min.		20,000xg/30 min.	
		Pellet	Supernatant	Pellet	Supernatant	Pellet	Supernatant
Anti-lymphocyte globulin (ALG)	Dounce homogenization Sonication NP40	% of cell-associated radioactivity ^{c)}					
		39.9	60.1	16.7	43.4	7.8	35.6
		5.2	94.8	12.7	82.1	28.0	54.1
		21.9	78.1	1.5	76.6	5.0	71.6
Anti-L-chain antibody	Dounce homogenization Sonication NP40	43.0	57.0	32.1	24.9	11.7	13.2
		6.6	93.4	8.1	85.3	35.0	50.3
		18.0	82.0	1.2	80.8	0.4	80.4
Tobacco mosaic virus protein (TMVP)	Dounce homogenization Sonication NP40	36.3	63.7	34.3	29.4	21.6	7.8
		6.5	93.5	5.6	87.9	46.8	41.1
		10.2	89.8	0.9	88.9	17.6	71.3

a) Lymphocytes were labeled with ALG (4 $\mu\text{g}/\text{ml}$), anti-L-chain antibody (560 ng/ml) or with TMVP (1.3 $\mu\text{g}/\text{ml}$) for 90 min. at 0°C as described in Chapter 2.

b) Labeled cells were Dounce homogenized (30 strokes) in 5 mM MgCl_2 /5 mM Tris-HCl, pH 7.4/15 mM NaN_3 , or sonicated for 60 seconds in 5 mM MgCl_2 /50 mM Tris-HCl, pH 7.4/100 mM NaCl/15 mM NaN_3 , or dissolved in the latter solution containing 0.5 % (v/v) NP40.

c) All values are averaged from the results of two experiments.

centrifugation.

Dounce homogenization was less effective in releasing the markers from insoluble cellular material (Table 4.2): 40 % of the total cell-associated label was removed in each case by centrifugation at 500g, and another 17-34 % by the 4,000g step. Thus, for cells labeled with ALG, anti-L-chain antibody or TMVP, the respective 4,000g supernatants contained only 43 %, 25 %, or 29 % of the cell-associated label. Except for ALG, however, most of this "soluble" label was sedimented during centrifugation at 20,000g.

The result of centrifuging the 4,000g supernatants of each of these cell extracts on density gradients is shown in Fig 4.9. There are several features which deserve comment:

- (a) The label in homogenates or sonicates of cells labeled with either ALG or anti-L-chain antibody was recovered in a band whose peak had a density of 1.12 g/cc.
- (b) Labeled material in NP40 extracts of anti-L-chain-labeled cells was recovered at a lower density (peak, 1.09 g/cc) than that of sonicated or homogenized cells. This indirectly supports the finding (Table 4.2) that NP40 fragments the plasma membrane more completely than do the other methods. Furthermore, the label from NP40-treated, ALG-labeled cells was recovered at a lower density (peak, 1.06 g/cc) than was that of anti-L-chain-labeled cells. Thus, it is clear that the detergent did not dissociate the antibodies, but that it resulted in the release of soluble complexes whose size was presumably related to the size of the antigens to which the antibodies were bound.
- (c) The distribution in density gradients of radioactivity from TMVP-labeled lymphocytes was quite distinct from that of the other two markers. Most of the label was recovered in denser portions (1.14 - 1.18 g/cc) of the gradients, even with NP40-extracts (Fig 4.9, bottom). Although TMVP must have bound only to the plasma membrane when incubated with intact cells, much of it may have bound only loosely and become dissociated upon cell disruption. Such material may have re-associated with denser intracellular material to produce the observed profiles. An alternative possibility is that ALG- and anti-L-chain-labeled plasma membrane antigens are preferentially lost from a plasma membrane "core", whose distribution is shown by TMVP. However, the fact that the radioactivity from Dounced or

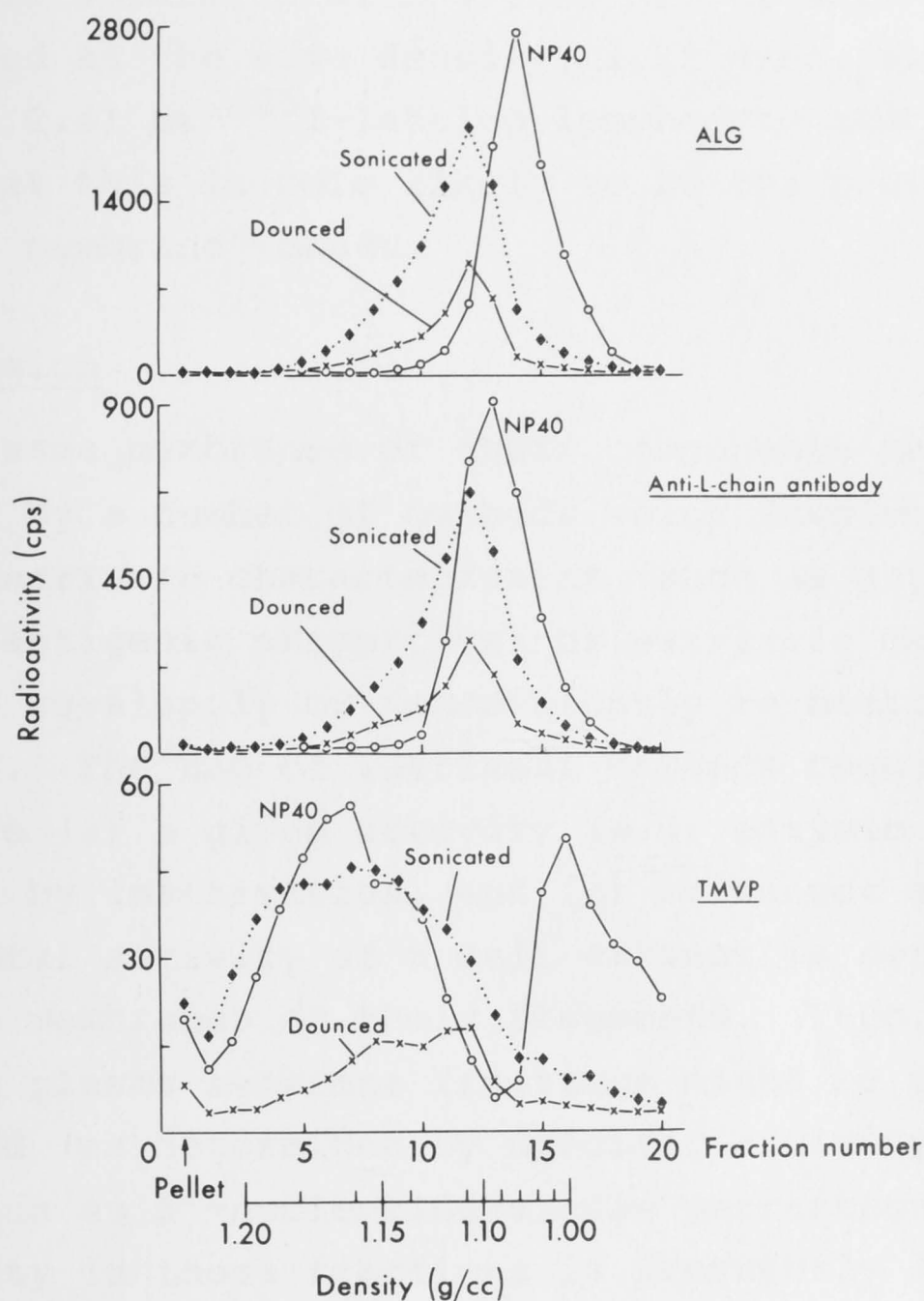


Figure 4.9: Radioactivity profiles resulting from density gradient centrifugation of the 4,000g supernatants of Dounce homogenized, sonicated, or detergent-treated lymphocytes which had been prelabeled with ^{131}I -ALG, ^{125}I -anti-L-chain antibody, or ^{131}I -TMVP

Sheep lymphocytes (1.8×10^9 cells in DRN^{*}) were divided into two 10 ml fractions of 9×10^8 cells each. To the first was added 13 μg of ^{131}I -labeled TMVP, and to the second, 5.6 μg of ^{125}I -labeled anti-L-chain antibody. After each suspension had been incubated for 60 min at 0°C , 5.6 μg ^{125}I -labeled anti-L-chain antibody and 40 μg of ^{131}I -labeled ALG was added to the first and second suspension respectively. After a further incubation of 90 min at 0°C , the cells were washed in DRN at $0-4^\circ\text{C}$, rinsed once in 5 mM MgCl_2 /100 mM NaCl /50 mM Tris-HCl , pH 7.5 and then each suspension was divided into 3 equal fractions of 3×10^8 cells each. The cells were sedimented and the pellets resuspended in 3 ml of (a) 5 mM MgCl_2 /5 mM Tris-HCl , pH 7.5; (b) 5 mM MgCl_2 /100 mM NaCl /50 mM Tris-HCl , pH 7.5; or (c) the latter buffer containing 0.5 % (v/v) NP40. They were then Dounce homogenized (a), sonicated (b), or given no further treatment (c). Each extract was centrifuged at 4,000g/15 min and the supernatants from this step were then centrifuged (SW25.3 rotor; 22,000 rpm, 18 h, 5°C) on 25-55 % (w/v) sucrose gradients containing the respective buffer.

*DRN: DBSS containing 10 % normal rabbit serum and 15 mM NaN_3 .

sonicated cells labeled with either ALG or anti-L-chain antibody was recovered at the same density, 1.12 g/cc, as 5'-nucleotidase activity (4.2.4) or ^{125}I -labeled lymphocyte membranes (4.2.2) suggests that this is more likely to be the position at which the "plasma membrane" bands.

4.3 Discussion

Plasma membranes or their components may be identified and assayed by a number of methods which involve the measurement of either *intrinsic* characteristics (such as physical, chemical, enzymic or antigenic properties) or *extrinsic* markers which can be attached covalently or noncovalently to membrane components (Table 4.3). The use of intrinsic markers requires particular care because (a) a given activity (e.g. enzymic or antigenic) may be lost by inactivation, and (b) it cannot always be assumed that the total activity of a cell extract is derived exclusively from plasma membranes or their fragments. Thus, although presumptive plasma membrane fractions might be enriched more than 20-fold (as determined by specific activity) in an enzyme activity such as 5'-nucleotidase, the percentage *recovery* of such activity in these fractions is frequently less than 10-20 % of the theoretical yield (e.g. Evans, 1971; Allan and Crumpton, 1970). This could arise if the yield of plasma membrane is very low, in which case a certain sub-population of the plasma membrane fragments might have been isolated; otherwise the 5'-nucleotidase may be located on other cellular structures and this enzyme may not be a suitable marker for the plasma membrane. If another marker, *known* to be *exclusive* to the plasma membrane, is recovered in a similar fashion, however, then the fractions containing the enzymic activity might reasonably be expected to be a representative sample of the plasma membrane, but in low yield (e.g. (Allan and Crumpton, 1970)).

In contrast to intrinsic activities such as discussed above, extrinsic radioactive plasma membrane markers cannot be "lost" by inactivation. Furthermore, they can in many instances (especially in the case of labeled proteins) be bound *exclusively* to plasma membrane components by incubation (frequently at 0°C) with intact cells before disruption of the latter. In this way plasma membrane components which are normally exposed to the

Table 4.3

Potential plasma membrane markers

TYPE OF MARKER		ADVANTAGES	DISADVANTAGES
INTRINSIC PROPERTIES	1. <u>PHYSICAL PARAMETERS</u> a) Morphology b) Chemical composition (RNA, sialic acid, cholesterol, etc.) 2. <u>ENZYMIC ACTIVITY</u> (e.g. 5'-Nucleotidase, ATPase, and others (e.g. adenylyl cyclase, alkaline phosphatase) on certain specialized cells 3. <u>ANTIGENIC ACTIVITY</u> (i.e. surface antigens)	*Qualitative assessment of membrane damage, etc. *Visualization of contaminants (e.g. mitochondria, lysosomes) - *Quantitative *May assay >1 surface component	*Subjective, nonquantitative *Possibility of artifacts - *Subject to inactivation by inhibition or denaturation *May not be exclusive to the plasma membrane *Can be blocked or denatured *May not be exclusive to plasma membrane
	1. <u>COVALENT</u> (e.g. iodine, sulphonic acid derivatives, etc.) 2. <u>NONCOVALENT</u> a) Antibodies (against ≥1 antigenic surface components) b) Biologically-active Substances (e.g. antigens, mito- gens, on lymphocytes; insulin, glucagon, on liver cells)	*May label plasma membrane exclusively *Quantitative *Cannot be inactivated *Assaying ≥1 component *Can bind exclusively to plasma membrane by labeling intact cells *Label can be quantitated and cannot be inactivated *May label and assay biologi- cally-active surface components	*May affect membrane properties *Antibodies may dissociate *Dissociated antibodies may readsorb to other cellular components after cell disruption *As for 2(a)

extracellular medium can be labeled, whereas other molecules of the same components which might be present intracellularly will not be labeled. The number of components which become labeled depends on the type of marker used. A substance which reacts covalently with proteins (e.g. Table 4.3, B.1), should label all proteins exposed on the external surface of the plasma membrane according to their size, frequency, and content and accessibility of groups with which the reagent reacts. Other markers (e.g. antibodies) can be made specific for selected membrane components or in some cases (e.g. antigens, hormones) can be used to label certain components which express an affinity for them. There is a possibility that noncovalently-bound markers may dissociate from the membrane components to which they initially bind. If this occurs the marker will not represent the membrane component(s) for which it was selected and it may even re-adsorb, specifically or nonspecifically, to intracellular components. The probability that such dissociation will occur depends on (a) the affinity of the marker for the substance to which it becomes attached, and (b) the conditions used in the isolation procedure.

The purpose of this study was to develop a procedure suitable for the isolation of plasma membranes from sheep lymphocytes. It was anticipated that once this was achieved, the components of the membrane could be separated and characterized. Components of the membrane which were (in the intact cell) exposed to the extracellular medium would be more easily identified, if beforehand the membrane could be exclusively or selectively labeled with ^{125}I . It was therefore important to determine, as was done with RBC (Chapter 3), whether the lymphocyte plasma membrane was labeled more selectively than intracellular material during radioiodination of intact cells.

^{125}I -labeled ALG and anti-L-chain antibody were considered to be suitable markers because each bound *exclusively* to the plasma membrane when incubated at 0°C with intact lymphocytes. Furthermore, each antibody bound specifically to certain antigenic components which were exposed on the "extracellular side" of the plasma membrane; ALG to an unknown number (but probably > 1) of non-Ig components; anti-L-chain antibody

to only Ig components (surface-Ig, Chapter 5). The use of these antibodies therefore provided a means for studying, during fractionation of cell extracts, the behaviour of two distinct populations of antigens derived exclusively from the plasma membrane. ^{131}I -Labeled (horse) anti-HeLa cell globulin has similarly been used to label HeLa cell plasma membranes (Boone, Ford, Bond, Stuart and Lorenz, 1969). As mentioned earlier, however, antibodies must remain associated with the antigens to which they initially bind if they are to be valid markers for membrane components. Although this will depend on the conditions which are used to disrupt and fractionate labeled cells, the absence of significant amounts of dissociated 7S antibody in cell extracts in this study suggests that dissociation did not occur to any marked degree. It is unlikely that the antibody dissociated after disruption of the cells and then re-associated with the same or other components.

In addition to providing a marker for plasma membrane components different from those labeled by ALG, anti-L-chain antibody was chosen for its ability to specifically label surface-Ig. A marker for surface-Ig molecules was important as they are likely to be, or to be associated with, the lymphocyte receptors for antigen (see Chapter 1). Other markers used for comparison with ALG and anti-L-chain antibody were (a) 5'-nucleotidase, a commonly-used intrinsic plasma membrane marker (e.g. Allan and Crumpton, 1970, 1971) and (b) tobacco mosaic virus protein (TMVP), which was used as an antigen. As is explained in Chapter 7, however, much of the cell-bound antigen is unlikely to have been bound to antigen-receptors on ABC specific for TMVP but rather to immunologically-unimportant sites on the surfaces of other lymphocytes.

The results of this study reveal several important features concerning the properties and isolation of plasma membranes from sheep lymphocytes. Firstly, cations were found to be important in determining the state in which the membrane material was isolated. EDTA, at low ionic strengths, so changed the membranes that the preparation lost opacity and the membrane fragments appeared to be more homogeneous but less dense. This effect was diminished, however, if the total salt concentration, including that of EDTA in the absence of other salts, was increased. Diminution of the EDTA effect was therefore due to

the substitution, in the membrane fragments, of chelated divalent cations by monovalent cations such as Tris^+ or Na^+ . Sodium and calcium have identical ion sizes and show isomorphous replacement in many minerals (Williams, 1970), and the replacement of Ca^{++} and Mg^{++} by Na^+ or K^+ has been observed in studies on rat liver microsomes (Sanui and Pace, 1967a,b). Consequently, the effect of EDTA at low net salt concentrations was considered to be due to the removal of cations from the membrane fragments. This could have caused (a) large fragments to disaggregate and/or (b) electrostatic repulsion and consequent expansion and hydration of polar and ionized membrane moieties. Such expansion could account for the observed decrease in density of the fragments.

The changes in membrane structure caused by exposure to EDTA should be considered when discussing the results of other workers who have isolated plasma membranes. Whilst some workers stabilize the membranes by exposing cells, prior to their disruption, to divalent ions such as Pb^{++} or Zn^{++} (Warren, Glick and Nass, 1966), others extract cells with isotonic buffers (Allan and Crumpton, 1970), with 1 mM NaHCO_3 (Evans, 1971), and McCollester (1970), reports isolating plasma membrane ghosts from Meth A cells using 0.2 mM EDTA/2.5 mM borate, pH 9.2. Considering the diverse ionic conditions that are employed in these and other studies, it may not be valid to compare the properties of membranes isolated in different studies without first determining how these properties are affected by changes in the ionic environment.

Secondly, the distribution of each marker in density gradients containing Mg^{++} was heterogeneous and although a peak was often observed at a density of 1.11-1.12 g/cc, a large proportion of the total marker was recovered at higher densities. The similar distribution of all the markers except TMVP, however, indicated that each marker (except TMVP) was indeed representing plasma membrane material. The difference observed using TMVP was probably due to its adsorption to intracellular material, for much of this protein is unlikely to have been bound to the plasma membrane as firmly as were either ALG or anti-L-chain antibody.

The combined use of differential and density gradient centrifugation indicated that in the presence of Mg^{++} the

membrane material was heterogeneous both in size and density. This heterogeneity could have originated from a number of sources. For instance the lymphocyte population was comprised of at least two different kinds of cell, Ig^+ and Ig^- (see Chapter 5). The plasma membranes of these cells were therefore distinct in their Ig content and possibly in other components and properties. Heterogeneous membrane fragments may also have resulted from vesicle formation, and/or the disintegration of the membranes into fragments of different compositions. In view of the similar distribution of ALG, anti-L-chain antibody and 5'-nucleotidase activity, it is unlikely that the latter was a major source of heterogeneity. In contrast, the formation of membrane vesicles could well have been a significant factor (Wallach and Kamat, 1964). Examination of cell extracts by electron microscopy and centrifugation on density gradients composed of polymers such as Ficoll might have determined the significance of this phenomenon.

It is also possible that heterogeneous membrane fragments were formed by aggregation with denser cellular material in the presence of Mg^{++} or at high (≤ 150 mM Cl^-) salt concentrations. 5'-Nucleotidase activity, as well as the radioactivity from cells labeled with ALG or anti-L-chain antibody, were each recovered with a peak at approximately 1.12 g/cc after centrifugation of Dounce homogenized or sonicated cells on density gradients. Furthermore, nearly all the radioactivity from ALG-labeled lymphocytes which were sonicated and treated with EDTA at low ionic strength before density gradient centrifugation, was recovered between 1.10 and 1.13 g/cc. The labeled material recovered at this density may therefore have been comprised of unaggregated fragments whose composition was representative of the intact plasma membrane. This seems likely when it is considered that gradient centrifugation of NP40-treated cells produced peaks of ALG or anti-L-chain antibody at lower densities than obtained with sonicated or homogenized cells. The fact that in the presence of NP40, ALG and anti-L-chain markers were recovered at different densities demonstrates that each antibody was bound to distinctly different cellular components, and that the material in Dounce homogenized or sonicated cells, even those treated with EDTA, is probably of a larger size than that in NP40 cell extracts. From this it might be concluded that fractionation of cells in EDTA at low ionic

strength is to be preferred to the use of buffers of high ionic strength or of those containing divalent cations. It would be interesting to determine by electron microscopy, and also by chromatography on Sepharose, the size and morphology of the material obtained under each condition.

Although this study did not result in the isolation of a "pure" preparation of sheep lymphocyte plasma membranes, it provided useful information on the fate of some membrane protein components under different conditions of isolation.

Firstly, radiolabeled antibodies specific for various antigenic membrane components have been shown to be valid and highly specific markers either for the plasma membrane, or more particularly for those membrane components to which the antibodies bind. Secondly, ionic conditions have been shown to be important in determining the state in which the membrane components are isolated. The use of EDTA at very low ionic strengths may be preferable for isolating a relatively homogeneous membrane preparation. Thirdly, the detergent Nonidet P-40 has been shown to disrupt the membranes without causing dissociation of antibodies from the membrane antigens to which they are attached. The size of the complexes released by treatment of labeled cells with NP40 seems to depend on the specificity of the antibody which is used to label the cells. Use is made of this phenomenon in Chapter 6.

Finally, the distribution in density gradients of TCA-insoluble radioactivity from sonicated ^{125}I -labeled lymphocytes was very similar to that of the other plasma membrane markers. This is suggestive but by no means conclusive evidence that radioiodination of intact sheep lymphocytes using chloramine-T preferentially labels plasma membrane rather than intracellular components. More conclusive evidence for this could be obtained by electron microscope autoradiography.

4.4 Summary

1. ^{125}I -Labeled antibodies have been used to label the plasma membranes of intact sheep lymphocytes. Evidence is provided which suggests that the antibodies are specific and valid markers for plasma membrane components.

2. Labeled lymphocytes were disrupted by Dounce homogenization, sonication, or by treatment with the detergent

Nonidet P-40. The fractionation of various plasma membrane markers was compared for cells disrupted by each of these methods, and under a variety of ionic conditions. Density gradient centrifugation of cells disrupted by homogenization, or sonication, in buffers of low ionic strength and containing EDTA resulted in recovery of antibody markers in high yield at an approximate density of 1.12 g/cc. Treatment of cells with NP40 did not cause the antibodies to dissociate from the membrane components to which they were bound, but did result in the release of soluble complexes whose size, and possibly density, seemed to depend on the identity of these components.

5.1 Introduction
5.2 Experimental results
5.3 Discussion
5.4 Summary

5.1 Introduction

The term 'T cell' is now widely used to describe a subset of lymphocytes which are involved in cell-mediated immunity and in some cases with the regulation of humoral responses. Similarly, bone marrow-derived, in mammals, or bursa-derived lymphocytes in birds, are termed 'B cells' and these are precursors of antibody-secreting cells (Boyd et al., 1969). Although the two types of cells cannot be readily distinguished by morphological criteria, B cells (but not T cells or thymocytes) have been shown in the mouse (Raff, 1970; Unanue et al., 1971) and chicken (Abell and

Chapter 5

Immunoglobulins on the surface of sheep lymphocytes:

Class and cellular distribution

This property has been used in the mouse as a means of identifying B lymphocytes. The Ig molecules exposed on the B cell surface are believed to be the receptors to which antigen is bound in the initial phase of the humoral response, but evidence supporting this view is indirect (Byrt and Ada, 1969; Warner, Byrt and Ada, 1970; Unanue, 1971a).

Lymphocytes possessing surface Ig determinants have also been found in the rabbit (Peralta, Peral and Amato, 1970; Davis, Paul, Sage and Goldfarb, 1971), rat (Kawman and Guilbert, 1971), man (Cooms, Feinstein and Wilson, 1969; Peralta, Peral and Amato, 1971; Gray, Abell and Pirofsky, 1971; Wilson and Mason, 1971) and sheep (De Vries, Weiss and Fox, 1972) and by analogy with the mouse, these cells are thought to be B lymphocytes. This chapter presents evidence for a similar population of lymphocytes in sheep.

5.2 Experimental results

5.2.1 Estimation of IgM and IgG on the surface of sheep lymphocytes

- 5.1 Introduction
- 5.2 Experimental results
- 5.3 Discussion
- 5.4 Summary

5.1 Introduction

The term 'T cell' is now widely used to describe thymus-derived lymphocytes which are involved in cell mediated immunity and in many cases with the regulation of humoral responses. Similarly, bone marrow-derived, in mammals, or bursa-derived lymphocytes in birds, are termed 'B cells' and these are precursors of antibody-secreting cells (Roitt *et al.*, 1969). Although the two types of cells cannot be readily distinguished by morphological criteria, B cells (but not T cells or thymocytes) have been shown in the mouse (Raff, 1970; Unanue *et al.*, 1971) and chicken (Rabellino and Grey, 1971; Kincade, Lawton and Cooper, 1971) to have large quantities of Ig determinants exposed on their surfaces. This property has been used in the mouse as a means of identifying B lymphocytes. The Ig molecules exposed on the B cell surface are believed to be the receptors to which antigen is bound in the initial phase of the humoral response, but evidence supporting this view is indirect (Byrt and Ada, 1969; Warner, Byrt and Ada, 1970; Unanue, 1971a).

Lymphocytes possessing surface-Ig determinants have also been found in the rabbit (Pernis, Forni and Amante, 1970; Davie, Paul, Mage and Goldman, 1971), rat (Avrameas and Guilbert, 1971), man (Coombs, Feinstein and Wilson, 1969; Pernis, Forni and Amante, 1971; Grey, Rabellino and Pirofsky, 1971; Wilson and Nossal, 1971) and amphibia (Du Pasquier, Weiss and Loor, 1972) and by analogy with the mouse, these cells are thought to be B lymphocytes. This chapter presents evidence for a similar population of lymphocytes in sheep.

5.2 Experimental results

5.2.1 Estimation of IgM and IgG on the surface of sheep lymphocytes

Using the radioimmunoassay procedure, the ability of lymphocytes and of IgM and IgG to neutralize both anti- μ or anti- γ -chain sera and thus to inhibit the precipitation of ^{125}I -labeled IgM or IgG was tested. The viability of cells following incubation with antisera at 4°C was $>90\%$, indicating that only surface and possibly secreted Ig would be measured. For total cellular Ig, cells were incubated with antisera in the presence of 1% NP40.

The total surface-IgM was calculated to be 5.5 ± 3.5 (mean ± 1 standard deviation) $\text{ng}/10^6$ cells (range 2.3 - 11.4) and total cellular IgM was usually slightly higher but never greater than twice this level. The total surface-IgG was calculated to be 1.2 ± 0.52 $\text{ng}/10^6$ cells (range 0.4 - 2.0), but the total content of the cells was 5 to 10 fold higher. This indicates that most of the cellular IgM was present on the cell surface, whereas IgG, although also on the surface, was present in greater amounts intracellularly.

5.2.2 Labeling of cells with ^{125}I -labeled antibody

5.2.2.1 Time course of labeling

Fig. 5.1 illustrates the rate of binding of ^{125}I -labeled anti- μ -chain antibody to lymphocytes as a function of time. If the amount bound to the cells after 4 h incubation is arbitrarily regarded as 100% (i.e. maximum labeling), then 56% of this maximum occurred within 30 min. and 70% after 60 min. A similar rate was observed for the labeling of mouse spleen cells by ^{125}I -labeled rabbit anti-mouse-L-chain antibody. In most subsequent experiments, cells were exposed to radiolabeled antibodies for 60 min.

5.2.2.2 Specificity of labeling

Artifacts in cell surface labeling work might arise either by phagocytosis of the labeled protein by cells or by 'nonspecific' labeling of cells due to the 'sticky' nature of the labeled protein. These factors were minimized by labeling cells at 0°C in the presence of both 15 mM NaN_3 , a metabolic poison, and a large excess of similar proteins (10% NRS).

Table 5.1 indicates the percentage of ^{125}I -labeled antibody (specific for μ , γ or L chains) bound to sheep

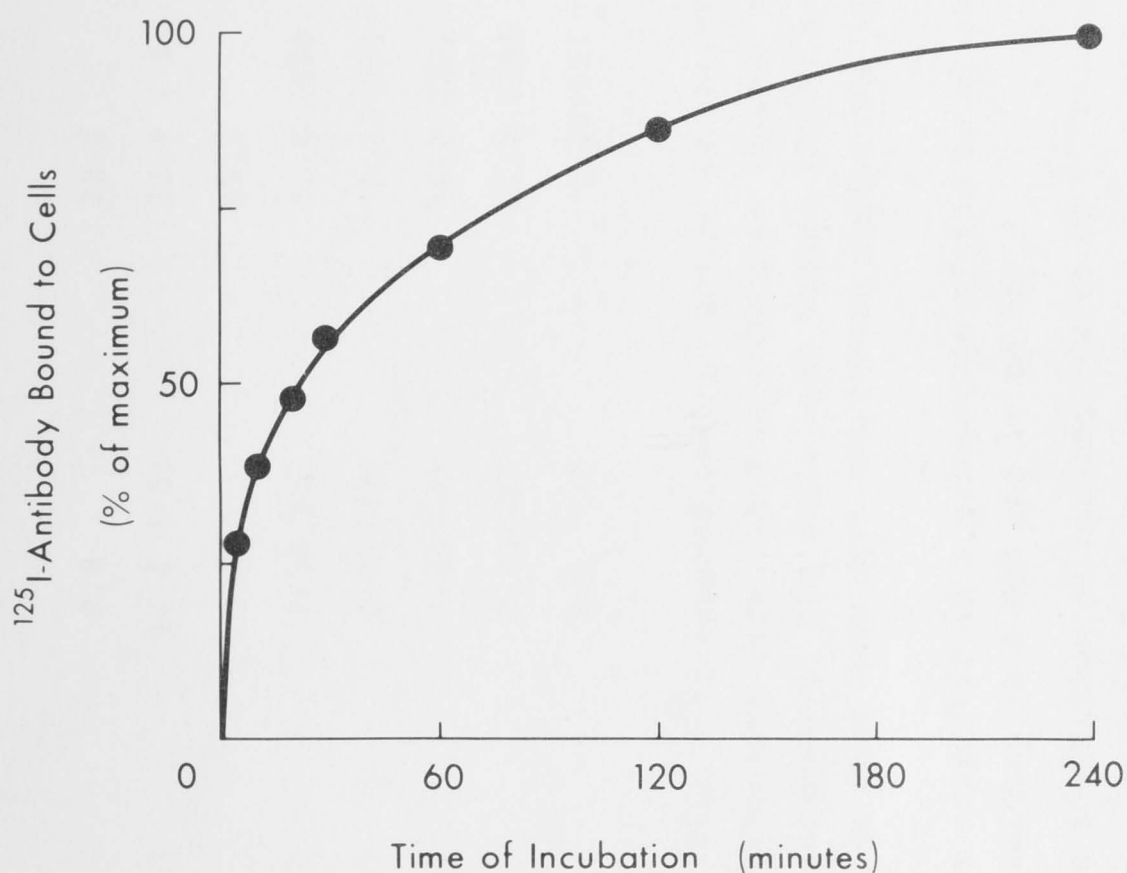


Figure 5.1: Time course of binding of ^{125}I -labeled anti- μ chain antibody to sheep lymphocytes. Lymphocytes (10^8 cells/ml) were incubated with anti- μ chain antibody (500 ng/ml) at 0°C in the presence of 10 % NRS and 15 mM NaN_3 in DBSS. After various periods of incubation, samples were removed and the cells were washed well at $0 - 4^\circ\text{C}$ in fresh medium. The radioactivity associated with the washed cell pellet was measured and is plotted as a percentage of that bound to the cells after 4 h incubation (arbitrarily 100 %). At this time (4 h), 24 % of the available antibody had bound to the cells.

Table 5.1

Specificity of binding of ^{125}I -labeled antibody to sheep lymphocytes^{a)}

<u>Antibody</u> <u>concentration (ng/ml)</u>	<u>Inhibitor</u> <u>($\mu\text{g/ml}$)</u>	<u>% Antibody bound to cells (% inhibition)</u>		
		<u>Anti-L chain</u>	<u>Anti-γ chain</u>	<u>Anti-μ chain</u>
50	-	18.1	9.8	22.0
250	-	17.0 (0)	10.4 (0)	23.9 (0)
1250	-	14.3	8.0	19.2
250	Sheep IgG (218)	0.47(97)	0.54(95)	21.6 (10)
250	Sheep IgM (150) ^{b)}	0.59(96)	1.91(82)	0.33(99)
250	Anti-L chain(30)	0.76(96)	4.80(54)	16.2 (32)
250	Anti- γ chain(52)	-	0.43(96)	21.7 (10)
250	Anti- μ chain(38)	-	9.0 (13)	1.28(95)

a) Lymphocytes (10^8 cells/ml) were incubated (0° , 60 min) with ^{125}I -labeled anti-L, anti- γ or anti- μ chain antibodies at each of the concentrations specified. Inhibitors were added as indicated to cell suspensions 5 min before the addition of labeled antibody. After incubation, the cells were washed (3x, 50 vol) and the cell pellets, together with triplicate samples taken from each cell suspension during the incubation, were measured for radioactivity.

The amount of antibody bound under these conditions is expressed as a percentage of the total exposed to the cells. The medium used throughout was DBSS containing 10 % NRS and 15 mM NaN_3 .

b) This IgM contained 1.2 % IgG (150 μg \equiv 1.8 μg IgG). This inhibition of binding (82 %) of ^{125}I -labeled anti- γ chain antibody by 150 $\mu\text{g/ml}$ of IgM was therefore expected from its estimated IgG content.

lymphocytes (60 min., 0°C), and the percentile reduction in this binding obtained in the presence of a number of inhibitors. Less than 1 % of labeled normal rabbit IgG or labeled anti-mouse-L-chain antibody was bound to sheep lymphocytes under similar conditions. At identical concentrations (e.g. 250 ng/ml), the percentage of ^{125}I -labeled anti-sheep-Ig antibodies bound was: anti- μ (24 %), anti-L (17 %), anti- γ (10 %). The binding was specific, as shown by the large degree of inhibition which occurred in the presence of excess of either unlabeled rabbit antibody or unlabeled sheep Ig.

Because labeled anti- μ -chain binding was slightly inhibited (10 %) by excess of unlabeled anti- γ -chain antibody and by unlabeled sheep IgG, subsequent incubations using labeled anti- μ -chain were done in the presence of unlabeled sheep IgG (>7 $\mu\text{g/ml}$). Similarly, labeled anti- γ -chain binding was performed in the presence of unlabeled sheep IgM (4 $\mu\text{g/ml}$). The presence of these Igs had little effect (<10 %) on the uptake of the ^{125}I -labeled anti-heavy-chain antibodies by the cells.

5.2.2.3 Incidence of labeled cells

Autoradiographs, prepared from suspensions of lymphocytes which had been exposed to ^{125}I -labeled antibody, were developed after 1-24 days exposure (see Fig. 5.2). Almost all labeled cells (>95 %) were small to medium-sized lymphocytes. A few polymorphs or blast-like cells were labeled but only slightly above background. Few cells (<1 %) were labeled significantly by labeled normal rabbit IgG, by anti-mouse-L-chain antibody or by anti-sheep-Ig antibodies in the presence of excess sheep Ig as inhibitor (Table 5.1).

In every preparation, lymphocytes could be described as either labeled or unlabeled (Table 5.2). This was so, even if long periods of exposure were used, when those cells labeled were hardly visible because of the dense clusters of silver grains over them.

It can also be seen (Table 5.2) that there was no significant difference in the incidence of cells labeled by each of the different anti-Ig chain antibodies. If the IgG and IgM determinants detected by the binding of these anti-

Figure 5.2

Autoradiographs of lymphocytes labeled with ^{125}I -labeled anti-sheep-Ig antibodies. Cells were labeled at 0°C with antibody (500 ng/ml, 60 min.), washed and smeared for autoradiography. The photographs shown are of smears exposed for 20 days, with the scale (20 μ) indicated. Cells were labeled with ^{125}I -labeled antibodies directed against sheep

- a) L chains
- b) γ chains
- or c) μ chains

The polymorph (arrowed) in (a) indicates the slight extent to which these cells were labeled compared to Ig^+ lymphocytes.

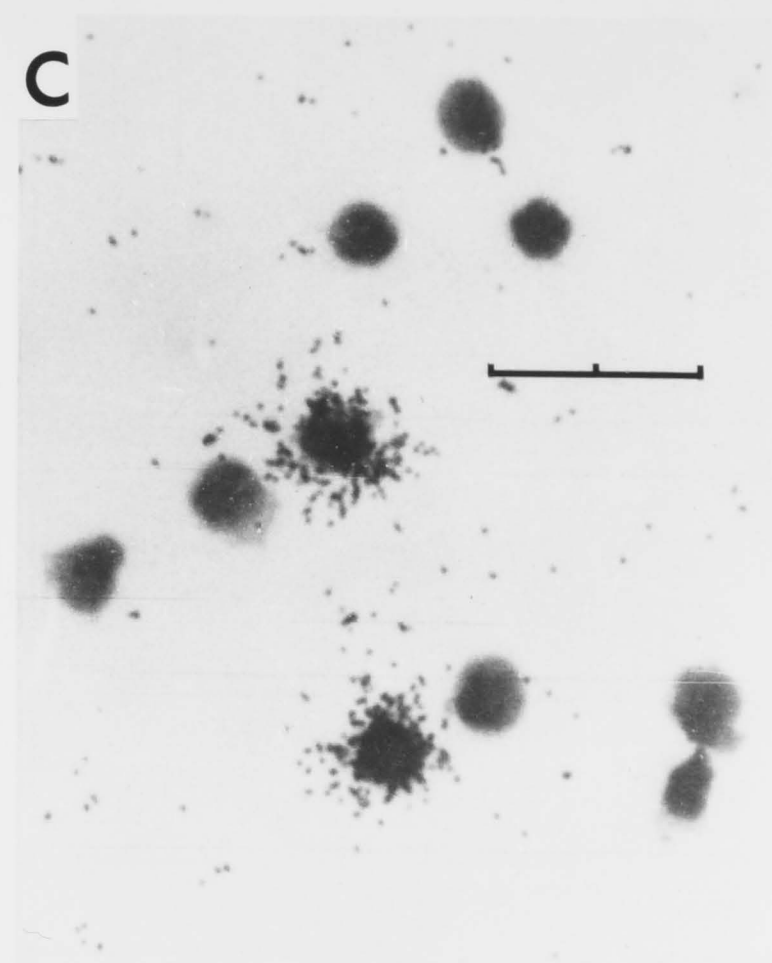
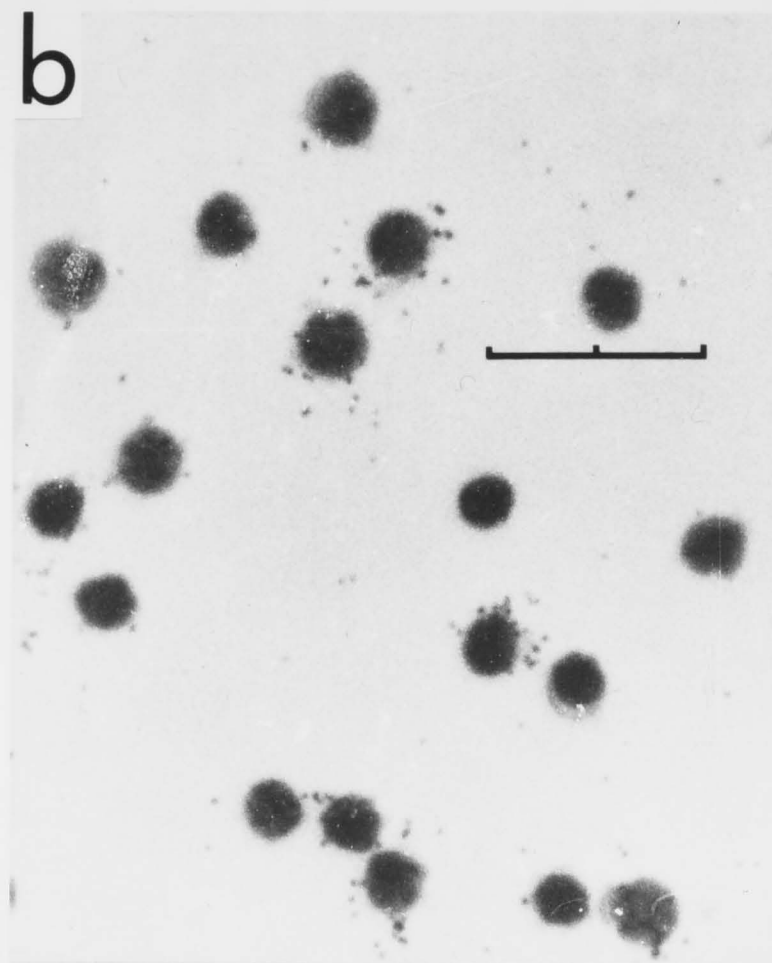
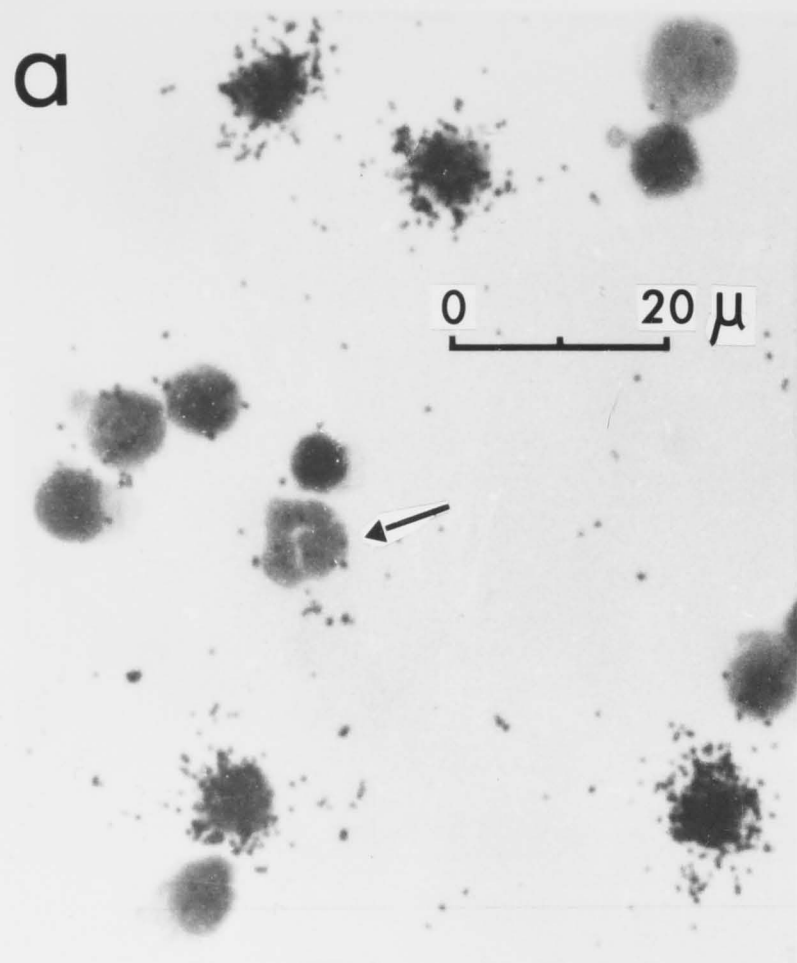


Table 5.2

Incidence of cells binding ^{125}I -labeled antibodies

<u>Antibody</u>	<u>Labeled lymphocytes^{a)}</u>	<u>Range</u>
Anti- μ chain	23.1 \pm 4.4	(19.8 - 27.3)
Anti- γ chain	17.7 \pm 1.0	(16.4 - 19.2)
Anti-L chain	20.4 \pm 1.5	(20.1 - 20.7)

- a) The mean percentage (± 1 standard deviation) of positive cells found in experiments on cell preparations from 3 sheep. Cells were incubated with ^{125}I -labeled antibody (500 ng/ml; specific activity, 1 $\mu\text{Ci}/\mu\text{g}$) in DBSS containing 10 % NRS and 15 mM NaN_3 (0° , 60 min), washed with the same medium and autoradiographs prepared from cell smears. Cells underlying more than 6 grains were scored positive. Most unlabeled cells (>90 %) had <1 grain/cell.

bodies to lymphocytes did not occur on the same cells, but on separate populations possessing either surface IgG or IgM (but not both), then the incidence of cells binding labeled anti-L-chain antibody should equal the sum of those binding either anti- μ or anti- γ -chain antibody alone. This is clearly not the case, suggesting that IgG and IgM occur *together* on the same cells.

Cells labeled most heavily with anti- μ -chain and least with anti- γ -chain antibody (Fig. 5.3). This order in the degree of cell labeling corresponded with both the amount of ^{125}I -labeled antibody which bound to the cells as measured by total radioactivity (Table 5.1), and also with the amount of IgG and IgM calculated to be present on the cell surface by radioimmunoassay (5.2.1).

5.3 Discussion

In this study, rabbit anti-Ig antibodies were used to demonstrate immunoglobulin determinants on the surface of sheep lymphocytes. The antibodies were not absorbed to the cells via their Fc moiety (cytophilic binding; Basten, Miller, Sprent and Pye, 1972) but bound specifically through their Fab combining sites, as only antibodies directed against sheep Ig chains showed binding and this could be blocked only in the presence of an excess of either the homologous unlabeled antibody or the Ig against which the antibody was directed. The binding of antibody to the lymphocytes was therefore a measure of Ig exposed on the cell surface.

It is not known whether or not this surface-Ig is synthesized by the cells on which it is detected, but the first possibility seems likely as sheep lymphocytes cultured for 3 days at 37°C in Ig-free medium still possessed surface-Ig as detected by radioimmunoassay. In addition, many mouse, rabbit or human lymphocytes which express surface-Ig do not simply absorb the Ig from the culture medium, and have in some studies been shown to synthesize their surface-Ig (Pernis, Forni and Amante, 1970; Johansson and Klein, 1970; Lerner, McConahey and Dixon, 1971; Vitetta and Uhr, 1972a,b; Melchers and Andersson, 1973).

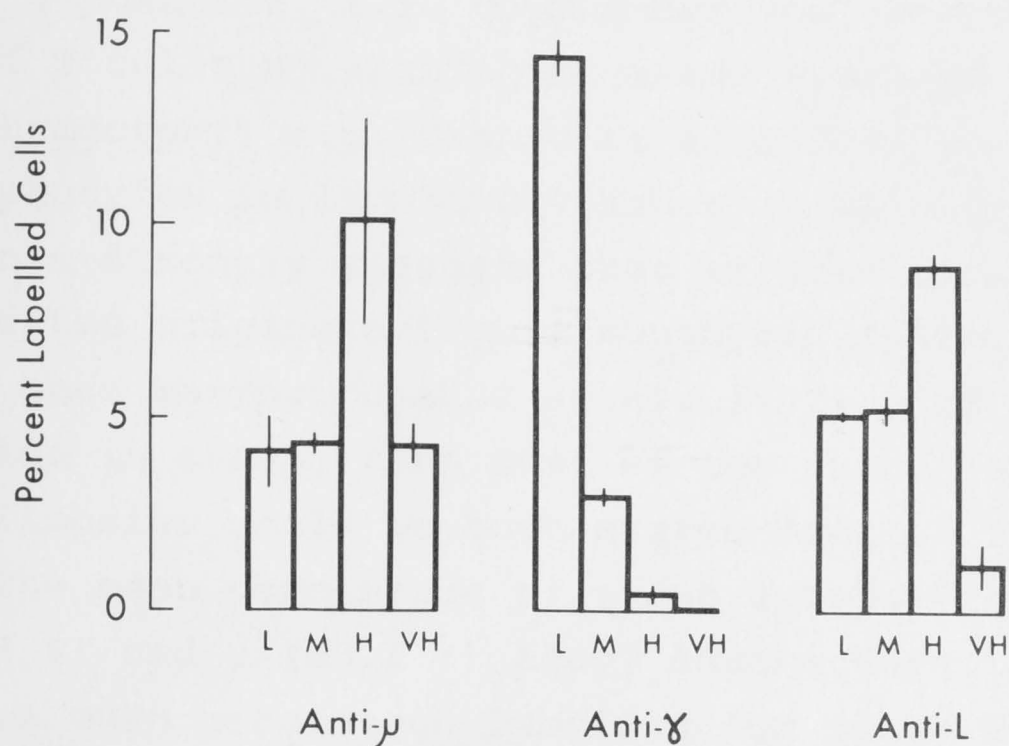


Figure 5.3: Incidence of cells binding ^{125}I -labeled rabbit anti-sheep-Ig antibodies to their surfaces.

Cells were incubated with labeled antibody (500 ng/ml, 2 h, 0°C), then washed, smeared and subjected to autoradiography (exposure, 20 days). The histogram shows the mean frequencies (± 1 standard deviation) of intact cells from 3 sheep which underlay different numbers of grains (6 to >50) after having been labeled with anti-Ig antibodies as indicated.

Categories estimated in terms of grains/cell were: 6 - 15 (L); 16 - 25 (M); 26 - 50 (H) and >50 (VH).

The incidence, in efferent sheep lymph, of lymphocytes labeling for surface-Ig (20 - 30 %) is similar to that found in cells from lymph nodes (13 - 23 %) and thoracic duct lymph (14 - 36 %) of mice (Raff, 1970; Bankhurst and Warner, 1971). Raff (1970) demonstrated that in the mouse, most lymphocytes which do not bind labeled anti-Ig antibodies are θ -positive, *i.e.* thymus-derived, and in mice depleted of T cells by artificial means (such as adult or neonatal thymectomy, *e.g.* Unanue *et al.*, 1971) the proportion of Ig⁺ lymphocytes in lymphoid tissues is markedly increased. This evidence strongly suggests that at least the majority of Ig⁺ lymphocytes originate from a nonthymic source, most likely the bone marrow (Unanue *et al.*, 1971). If this is also the situation in sheep, then most of the cells labeled by anti-Ig antibodies would be bone marrow-derived B lymphocytes.

The mean percentage of sheep lymphocytes labeling for γ (17.7 %) and μ (23.1 %) heavy chains totalled 40.6 %. However, the mean percentage labeling for light chains was only 20.4 %. If γ and μ chains are present as Igs, then most of the labeled cells must have had both IgG and IgM on their surfaces. Other evidence implying the presence of single or multiple Ig classes on the surface of antibody-secreting or precursor cells is contradictory. That individual cells may possess multiple Ig classes on their surface is implied by a number of studies, including the transformation of rabbit lymphocytes by anti-Ig sera (Sell, Lowe and Gell, 1970), the demonstration of more than one Ig class in single human myeloma cells (Costea *et al.*, 1967) and in single lymphoid cells in culture (Takahashi *et al.*, 1968), and by the binding of labeled anti-Ig antibodies to mouse lymphoid cells (Bankhurst and Warner, 1971; Nossal *et al.*, 1972). On the other hand, evidence that cells possess only one Ig class on their surface is provided by studies on the binding of labeled anti-Ig antibodies to lymphocytes from the mouse, rabbit and man (Rabellino *et al.*, 1971; Jones, Torrigiani and Roitt, 1971; Pernis, Forni and Amante, 1970, 1971; Grey, Rabellino and Pirofsky, 1971; Fröhland and Natvig, 1971). Considering that many human lymphoma cells bear IgM, IgG, IgA and β_2 -globulin (a serum complement component) on their surface when initially

biopsied, but only IgM after serial passage *in vitro* or *in vivo* in congenitally athymic mice (Klein *et al.*, 1968; Povlsen *et al.*, 1973), it is clear that involvement of cytophilic serum Ig must be carefully evaluated in future work if these differences are to be resolved.

Based on results obtained by radioimmunoassay, it was calculated that lymphocytes from sheep efferent lymph have on their surface 1.2 ± 0.52 ng IgG and 5.5 ± 3.5 ng IgM equivalents per 10^6 cells. If it is considered that only 20 % of these lymphocytes were labeled by anti- μ or anti- γ -chain antibodies, then these levels are equivalent to $19,200 \pm 8,200$ molecules IgG and $14,700 \pm 9,300$ molecules IgM (19S, or $73,600 \pm 46,800$ 7S molecules) per labeled cell. Much of this IgM seems to be present as a 7S, rather than a 19S molecule (Chapter 6), as has been found for the surface-IgM of lymphocytes from mice (Vitetta, Baur and Uhr, 1971; Vitetta and Uhr, 1972a,b; Marchalonis, Cone and Atwell, 1972; Melchers and Andersson, 1973; Parkhouse, 1973) and man (Eskeland, Klein, Inoue and Johansson, 1971). The number of Ig molecules estimated to be present on sheep lymphocytes labeled by anti-Ig antibodies agrees well with estimates for cells from other sources. Human leukemia cells were reported by Eskeland *et al.* (1971) to possess approximately 25 ng surface-IgM/ 10^6 labeled cells, compared to 27.5 ng/ 10^6 labeled sheep cells calculated here. Unanue *et al.* (1971), using mouse spleen cells to inhibit the precipitation of ^{125}I -labeled IgG by anti- κ -chain serum, reported 0.2 - 1.0 ng (Ig) N/cell, which, assuming 50 % of the cells were labeled, is equivalent to 10,000 - 54,000 molecules 7S Ig/labeled (Ig⁺) cell. Other estimates by the same group (Rabellino *et al.*, 1971; Grey, Colon, Campbell and Rabellino, 1972) attribute 50,000 - 150,000 molecules 7S Ig per labeled mouse spleen cell. Although variation is evident, these values lie within a 2- to 3-fold range and provide approximate quantitations on the Ig exposed on the surface of these cells. This quantity seems to be similar for human, mouse and sheep Ig⁺ lymphocytes.

5.4 Summary

Lymphocytes obtained by cannulation of the efferent

duct of sheep popliteal nodes were incubated *in vitro* with ^{125}I -labeled antibodies. The percentage of lymphocytes labeling for μ , γ or light chains was in each case approximately 20 - 25 %, but cells were most heavily labeled by anti- μ -chain and least by anti- γ -chain antibodies. As the sum of the proportions of cells labeling for μ and γ chains (total, 40.6 %) greatly exceeded that labeling for light chains (20.4 %), it was concluded that labeled cells had both γ and μ chains exposed on their surfaces, probably as IgG and IgM.

Lymphocytes were titrated against anti- μ and anti- γ -chain sera and means (\pm 1 standard deviation) of 19,200 \pm 8,200 molecules IgG and 14,700 \pm 9,300 molecules IgM (19S, or 73,600 \pm 46,800 7S molecules) were calculated to be exposed on the surface of each cell labeled by anti-Ig antibodies.

It is suggested that those cells from the sheep which bound anti-Ig antibodies were bone marrow-derived lymphocytes as has been found in the mouse.

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6.1 Introduction

There is now considerable evidence for the existence of surface-Ig on avian spleen-derived lymphocytes, or their mammalian bone marrow-derived equivalents. It seems certain that the greater proportion of this is IgM, mostly on small lymphocytes, although other Ig classes have been demonstrated. This has been shown using mainly fluorescent or 125 I-labeled anti-Ig antibodies to label normal lymphocytes from the mouse (Takahashi et al., 1971; Gumpert et al., 1971; Bankhurst and Warner, 1971; Weiss and Davis, 1971; Zet (Avrameas and Gilbert, 1971), rabbit (Parris, Ford and Asanuma, 1970, 1971), sheep (Chapter 3), and (Cooke, Falciani and

Chapter 6

Immunoglobulins on the surface of sheep lymphocytes:
Class, size and fate during incubation of lymphocytes
at 37°C

Surface-(7S)-IgM on lymphocytes has been demonstrated on lymphocytes from humans suffering chronic lymphocytic leukaemia (Klein, Isaac and Johnson, 1971). Furthermore, biosynthetic studies (Vitetta and Uhr, 1972a,b; Melchers and Andersson, 1973; Parkhouse, 1973) and lactoperoxidase-catalysed radioiodination of the cell surface (Vitetta, Uhr and Uhr, 1971; Vitetta and Uhr, 1972a,b; Melchers, Cone and Averbach, 1972) have indicated that the surface-IgM on normal mouse lymphocytes also consists of 7S molecules which, it is claimed, are released slowly into the medium, possibly still attached to some membrane components, and replaced in the membrane by newly-synthesized Ig (Melchers, Cone and Averbach, 1972; Vitetta and Uhr, 1972a,b).

The importance of lymphocyte surface-Ig is its presumed role as a receptor for antigen (Bytt and Ada, 1969; Warner, Bytt and Ada, 1970), at least on B cells. There has therefore been considerable interest in the class, size and surface distribution of these molecules and their turnover by the lymphocyte. When mouse lymphocytes are labeled with anti-Ig antibodies there is a rapid, partial loss of label into the medium at 37°C, whilst the remainder is maintained by the

- 6.1 Introduction
- 6.2 Experimental results
- 6.3 Discussion
- 6.4 Summary

6.1 Introduction

There is now considerable evidence for the existence of surface-Ig on avian bursa-derived B lymphocytes, or their mammalian bone marrow-derived equivalents. It seems certain that the greater proportion of this is IgM, mostly on small lymphocytes, although other Ig classes have been demonstrated. This has been shown using (mainly) fluorescent or ^{125}I -labeled anti-Ig antibodies to label normal lymphocytes from the mouse (Takahashi *et al.*, 1971; Unanue *et al.*, 1971; Bankhurst and Warner, 1971; Wilson, Nossal and Lewis, 1972), rat (Avrameas and Guilbert, 1971), rabbit (Pernis, Forni and Amante, 1970, 1971), sheep (Chapter 5), man (Coombs, Feinstein and Wilson, 1969; Wilson and Nossal, 1971) and chicken (Rabellino and Grey, 1971; Kincade, Lawton and Cooper, 1971).

Surface-(7S)-IgM has been demonstrated on lymphocytes from humans suffering chronic lymphocytic leukemia (Eskeland, Klein, Inoue and Johansson, 1971). Furthermore, biosynthetic studies (Vitetta and Uhr, 1972a,b; Melchers and Andersson, 1973; Parkhouse, 1973) and lactoperoxidase-catalyzed radioiodination of the cell surface (Vitetta, Baur and Uhr, 1971; Vitetta and Uhr, 1972a,b; Marchalonis, Cone and Atwell, 1972) have indicated that the surface-IgM on normal mouse lymphocytes also consists of 7S molecules which, it is claimed, are released slowly into the medium, possibly still attached to some membrane components, and replaced in the membrane by (presumably) newly-synthesized Ig (Marchalonis, Cone and Atwell, 1972; Vitetta and Uhr, 1972a,b).

The importance of lymphocyte surface-Ig is its presumed role as a receptor for antigen (Byrt and Ada, 1969; Warner, Byrt and Ada, 1970), at least on B cells. There has therefore been considerable interest in the class, size and surface distribution of these molecules and their turnover by the lymphocyte. When mouse lymphocytes are labeled with anti-Ig antibodies there is a rapid, partial loss of label into the medium at 37°C , whilst the remainder is endocytosed by the

labeled cell (Wilson, Nossal and Lewis, 1972). The latter process, correlated with "capping" of the surface-bound label (Taylor *et al.*, 1971; Loor, Forni and Pernis, 1972; Elson, Singh and Taylor, 1973), has been observed by electron microscopy (Unanue, Perkins and Karnovsky, 1972a,b; de Petris and Raff, 1972) and by bulk loss of antibody from the cell surface but not from the cell (Wilson, Nossal and Lewis, 1972). Capping and endocytosis are both markedly temperature dependent and are inhibited by metabolic poisons, but their significance is unknown (Andersson, Sjöberg and Möller, 1972). It is noteworthy that capping, induced by antibodies binding to cell surface-antigens, is not restricted to lymphoid cells but has been observed in numerous other cell types (*e.g.* Leonard, 1973).

This chapter presents further data concerning the nature of the surface-Ig of sheep lymphocytes and of the metabolic fate of complexes of these molecules. A technique was used by which cells were labeled with ^{125}I -labeled antibody, dissolved in detergent and the extracts analyzed by sedimentation centrifugation. By this procedure, the size of (surface-Ig/anti-Ig) complexes was determined and the metabolic nature of each studied by treating labeled cells in various ways before dissolving them. The capacity of lymphocytes to bind labeled anti- μ or anti- γ -chain antibody after various times of incubation at 0°C or 37°C , and the concentration of sheep IgG and IgM in the culture medium, were also estimated.

6.2 Experimental results

6.2.1 Nature of complexes formed by binding of labeled antibody to the lymphocyte surface

6.2.1.1 Complexes formed with IgG antibodies

To determine the nature of complexes formed at the cell surface by the binding of labeled antibody, lymphocytes were labeled with either anti-L-chain antibody or ALG, washed and dissolved in an NP40 solution. The size of radioactive complexes in the soluble extracts was determined by sedimentation. Fig. 6.1a shows that the label in extracts from anti-L-chain-labeled cells sedimented in two peaks, one

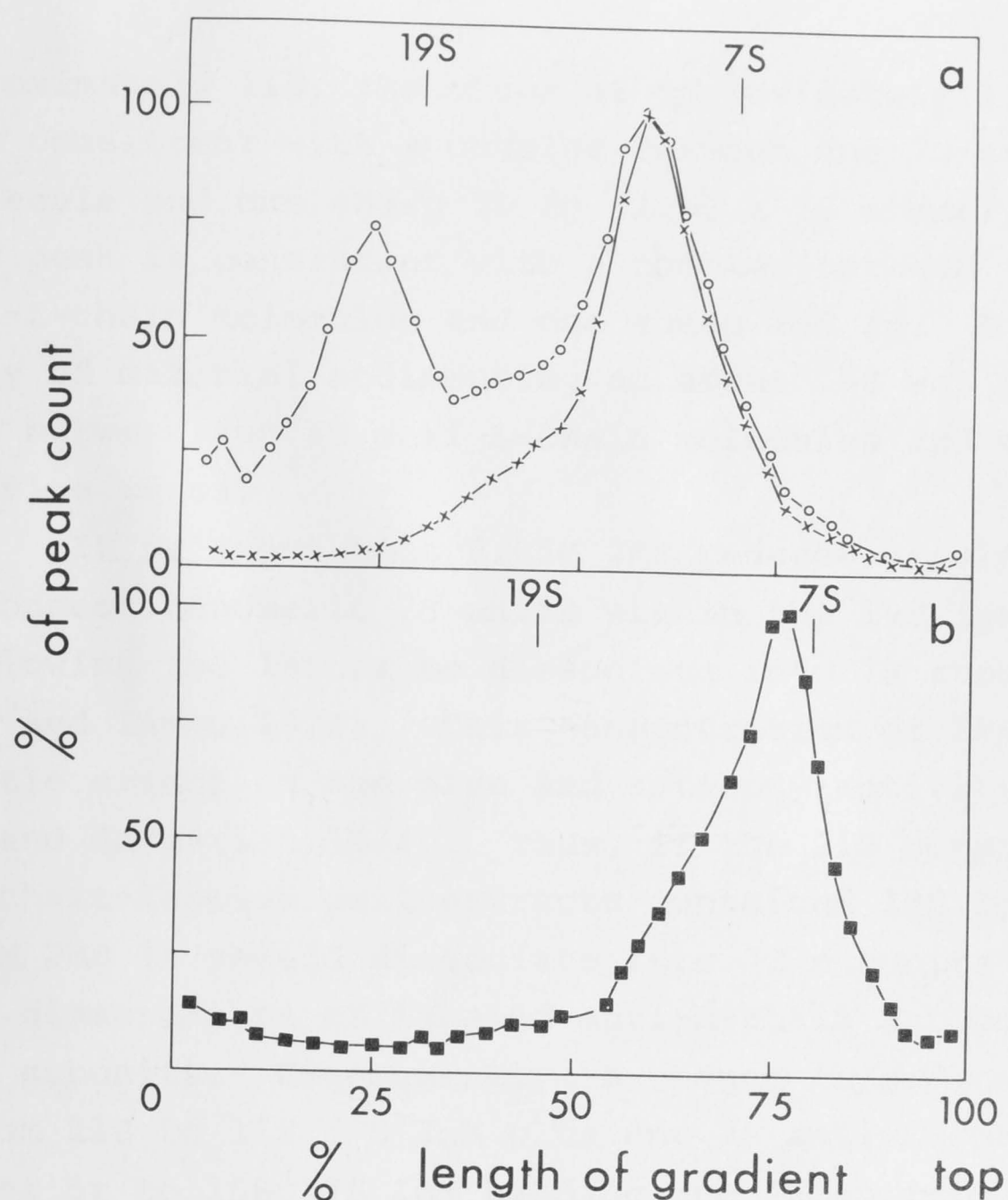


Figure 6.1: Sedimentation pattern of radioactive components present in NP40 extracts of lymphocytes labeled with either ^{125}I -labeled anti-L chain antibody (a) or ALG (b).

Cells, in DBSS containing 10 % NRS and 15 mM NaN_3 , were labeled with antibody (50 ng/ml, 0°C , 2 h), washed at $0 - 4^\circ\text{C}$ and then resuspended in 0.4 % NP40/75 mM NaCl/75 mM tris-HCl, pH 7.5/15 mM NaN_3 . After 60 min at 0°C , the extracts were centrifuged (750xg, 10 min) to remove insoluble material and the supernatants were sedimented through gradients of sucrose (10 -30 %, w/v) dissolved in the NP40 extraction solution (above).

One gradient in (a), (X — X), also contained 2-mercaptoethanol (0.05 M). The positions of 7S and 19S markers are indicated.

Peak counts were: (a) X — X 483 cps
 O — O 278 "
 (b) ■ — ■ 169 "

at approximately 11S, the other at approximately 21S. The 11S peak is consistent with a complex between one 7S anti-L-chain IgG molecule and one sheep 7S Ig (*i.e.* a 7S dimer), whereas the 21S peak is consistent with a complex between one or two 7S anti-L-chain molecules and one sheep 19S Ig. A smaller quantity of material sedimenting at about 15S was presumably a complex between two 7S anti-L-chain molecules and one 7S sheep Ig, or vice versa.

It is known that 0.05M 2ME reduces disulphide bonds which connect monomeric 7S units within the 19S IgM pentamer, thus allowing the latter to dissociate into 7S subunits (Mukkur and Inman, 1970). This concentration of 2ME, however, has little effect on the size and antibody activity of 7S IgG (Schur and Christian, 1964). Thus, if the 21S component from anti-L-chain-labeled cell extracts contained 19S IgM, then in 0.05M 2ME it should dissociate into 7S subunits *without* causing dissociation of labeled anti-L-chain antibody from labeled subunits. Consequently, a change in sedimentation rate from 21S to 11S (7S IgM plus one 7S anti-L-chain IgG molecule) or to 15S (7S IgM binding two 7S anti-L-chain molecules, or vice versa) would be expected. When this was done (Fig. 6.1a), the radioactivity was recovered as an 11S component, supporting the conclusion that the 21S complex contains 19S sheep IgM.

When extracts of cells labeled with anti- μ -chain IgG were analyzed, a sedimentation profile almost identical to that of extracts from anti-L-chain-labeled cells was obtained, *i.e.* both a 21S (2ME-sensitive) and an 11S peak were apparent. In contrast, sedimentation of extracts from cells labeled with anti- γ -chain IgG revealed labeled material up to 15S including a peak at 11S, but no 21S component. Because of the previous demonstration of the specificity of these anti-Ig reagents for cell surface-Ig (Chapter 5), it seemed certain that there were at least two sizes of IgM, 19S and 7S, present on these cells. IgG is present in much smaller quantities than IgM, (Chapter 5).

It is noteworthy that extracts from cells labeled with ALG showed only a large peak at about 9-10S, a declining amount of material up to approximately 15S and no 21S peak (Fig.

6.1b). This sedimentation profile presumably represents a variety of antigenic membrane components (of unknown nature), each attached to its respective (labeled) antibody molecule.

In subsequent experiments a significant proportion of cell-bound antibody occurred in dissociated (7S) form during sedimentation in NP40. This probably reflected the presence of low affinity antibodies which may have bound to cells at 0°C because higher concentrations of antibody were used. At 37°C, or in the presence of NP40, low affinity antibodies are more likely to dissociate from their substrates than are high affinity molecules.

6.2.1.2 Complexes formed with anti- μ -chain Fab

Although it seemed certain from the above results that both 19S and 7S IgM were present on the sheep lymphocyte surface, there remained the possibility that the 21S and 11S complexes found in cell extracts were artifacts caused by cross-linking (aggregation) of smaller complexes via the divalent antibody. One way of eliminating this possibility was to use a monovalent reagent. Thus the Fab fragment of anti- μ -chain IgG was used to label sheep lymphocytes. This reagent, in addition to being monovalent, is smaller than IgG. Therefore, (Fab/antigen) complexes should (a) not cross-link and (b) be more nearly the size of the substrate molecules to which the Fab binds (IgM or μ chains, in this instance). This is clearly demonstrated in Fig. 2.8, where complexes between serum Ig and anti-L, anti- γ or anti- μ -chain Fab sedimented at 7-8S or 19S.

The result of sedimenting NP40 extracts from cells labeled with anti- μ -chain Fab is shown in Fig. 6.2. Two radioactive complexes, at 19S and at 7-8S, are clearly discernible. This further supports the conclusion that both 19S and 7S IgM are present on the cell membrane. It can be seen that a large proportion of the cell-associated Fab was dissociated by the detergent, resulting in a peak at 3.5S.

6.2.2 Loss of labeled antibody from lymphocytes

At 0°C, cells which had bound labeled antibodies retained most of their radioactivity (>90 %). However at 37°C a rapid loss occurred, as shown in Fig. 6.3. Within 30

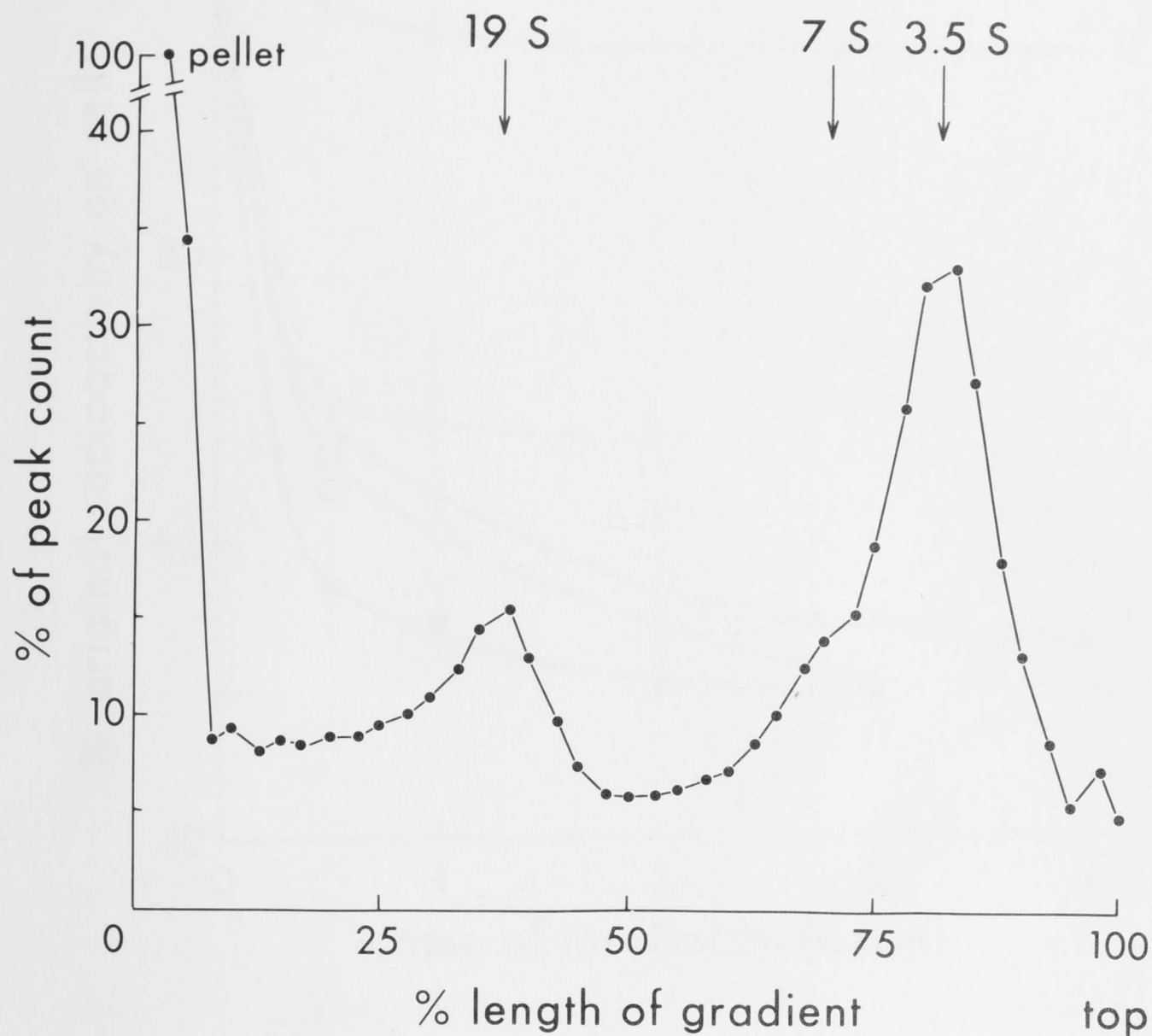


Figure 6.2: Radioactivity profile obtained after sedimentation of an NP40 extract of sheep lymphocytes pretreated with ^{125}I -labeled anti- μ chain Fab.

Lymphocytes were labeled with the monovalent antibody fragment (300 ng/ml, 75 min, 0°C) in DBSS containing 10 % NRS and 15 mM NaN_3 , washed and then dissolved in 0.1 % NP40/5 mM EDTA/5 mM tris-HCl, pH 7.5. After 60 min at 0°C , the extract was centrifuged (750xg, 10 min) and the supernatant sedimented through a 10 - 30 % (w/v) sucrose gradient containing the NP40 extraction buffer (above) (SW50.1 rotor; 45,000 rpm, 5 h, 5°C). The peak count was 123 cps.

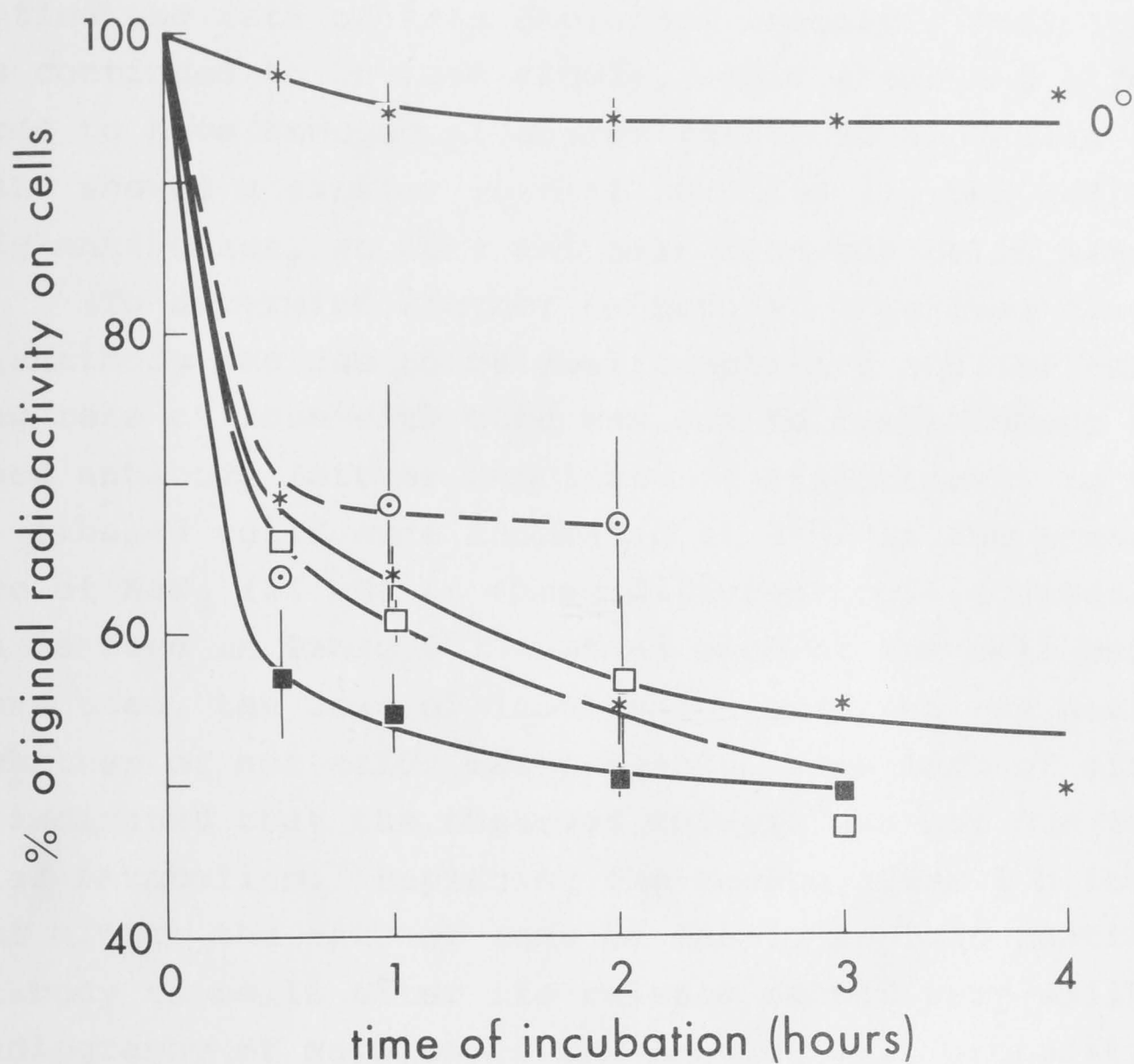


Figure 6.3: Loss of radioactivity at 37°C by lymph cells prelabeled at 0°C with various ^{125}I -labeled antibodies.

Cells were incubated with labeled antibody (500 ng/ml, 60 min, 0°C) in DBSS/10 % NRS/15 mM NaN_3 , washed and then resuspended in DBSS containing 10 % FCS at concentrations ranging between 1 and 5×10^7 cells/ml in 4 separate experiments. They were incubated at either 0°C or 37°C and at the indicated times, samples were removed, the cells pelleted (750xg, 5 min) and the radioactivity measured in both the cell pellets and the supernatant media. Points plotted are mean values and vertical bars represent twice one standard deviation. ^{125}I -Labeled antibodies used to label the cells were:

- | | |
|-------------------------|-------------|
| a) anti- μ chain | * — * — * |
| b) anti- γ chain | □ — □ — □ |
| c) anti-L chain | ■ — ■ — ■ |
| d) ALG | ○ -- ○ -- ○ |

min., 30-40 % of the label was released into the medium, after which time the rate of loss decreased sharply. Anti-Ig antibodies continued to be lost slowly, until after 2-3 h loss appeared to have stopped at approximately 50 %. Cells labeled with ALG showed a similar initial loss (30 %), but unlike the anti-Ig antibodies, no more was lost from the cells after 1 h.

To determine whether or not (a) this loss of surface-bound antibody was due to metabolic activity and (b) the slowing rate of loss with time was due to reattachment of released antibody (either complexed or dissociated) to the cells, labeled cells were incubated at 37°C in the presence or absence of NaN₃ (15 mM) at three different cell concentrations. It can be seen in Table 6.1 that at each of the cell concentrations used, the loss of label which occurred was nearly the same whether or not azide was present. This lack of effect by azide indicated that the observed release was not due to cellular metabolism. Replacing the medium after 1 h incubation did not affect the rate of loss of label, so that reattachment of antibody to cells after its release seemed very unlikely. Autoradiography of samples of the labeled cell preparations taken during incubation showed that mean grain counts/cell decreased while the proportion of labeled cells remained relatively constant.

6.2.3 Nature of Ig complexes released or retained by labeled cells incubated at 0°C or 37°C

Because of the loss at 37°C of radioactivity from cells labeled with anti-Ig antibody, the size of radioactive components in released material, and in that retained by cells, was determined. Anti-L-chain-labeled cells were incubated either at 0°C, or at 37°C in the presence or absence of 15 mM NaN₃. After selected periods, the cells were dissolved in NP40 and the extracts and incubation supernatants were subjected to sedimentation. The results shown in Fig. 6.4a are for cells incubated for 2 h before extraction. Total cell-associated radioactivity was less after incubation at 37°C than after incubation at 0°C because of loss into the medium (see 6.2.2). To enable the peaks in different extracts to be compared, all radioactive counts were therefore standardized (Fig. 6.4).

Table 6.1

Effect of cell concentration and azide (15 mM) on the loss at 37°C of radioactivity from lymphocytes prelabeled at 0°C with ¹²⁵I-labeled anti-μ chain antibody

		<u>Incubation time</u> (hours)				
		<u>0</u>	<u>0.5</u>	<u>1.0</u>	<u>2.0</u>	<u>4.0</u>
<u>Cell concentration</u> (cells/ml)		<u>Cell-associated radioactivity (%)</u>				
10 ⁵	+ azide ^{b)}	100	62	55	42	42
	- azide	100	58	54	42	41
10 ⁶	+ azide	100	67	60	51	45
	- azide	100	67	59	47	41
10 ⁷	+ azide	100	70	62	55	50
	- azide	100	69	61	53	46

a) Lymphocytes, prelabeled with ¹²⁵I-labeled anti-μ chain antibody (500 ng/ml, 60 min, 0°C) and washed at 0-4°C, were incubated at the indicated concentrations in DBSS supplemented with 10 % FCS and also with 15 mM NaN₃ where stated. At the indicated times, samples were withdrawn, diluted 1/3 and centrifuged to sediment the cells. Both the cell pellet and the supernatant medium were measured for radioactivity. Cells kept at 0°C retained >90 % of their radioactivity during the 4 h incubation.

b) Radioactive counts at 10⁵ cells/ml were low and therefore less reliable than at higher cell concentrations.

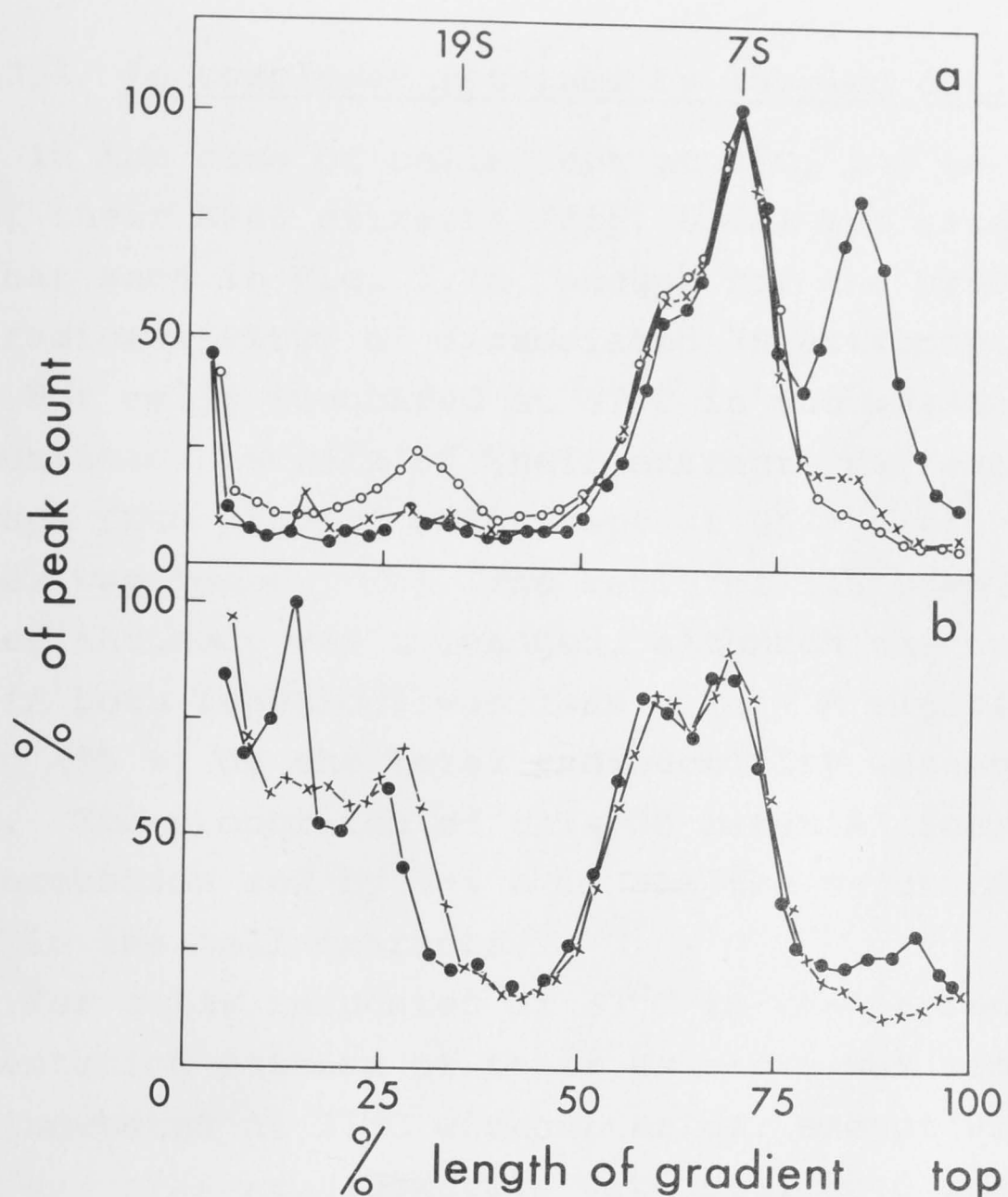


Figure 6.4: Sedimentation of (a) cell extracts and (b) incubation supernatants from lymphocytes labeled with anti-L chain antibody.

Lymphocytes (10^8 cells/ml) were incubated with ^{125}I -labeled anti-L chain antibody (450 ng/ml, 60 min, 0°C) in DBSS/10 % NRS/15 mM NaN_3 , washed, and divided into 3 equal fractions before being incubated at 1×10^7 cells/ml in DBSS containing 10 % FCS, with or without 15 mM NaN_3 at 0° or 37°C (see below). After 2 h incubation, the cells were sedimented, dissolved in 0.5 % NP40/5 mM EDTA/5 mM Tris-HCl, pH 7.5/15 mM NaN_3 and kept at 0°C for 60 min. They were then centrifuged ($1000\times g$, 15 min, 4°C). The resulting supernatants (cell extracts) and the original incubation supernatants were each sedimented (SW50.1 rotor; 45,000 rpm, 7 h, 10°C) through 10-30 % (w/v) sucrose gradients containing either 0.1 % NP40/5 mM EDTA/5 mM Tris-HCl, pH 7.5 (extracts) or DBSS (incubation supernatants).

	Cells incubated at:	Peak count (cps)
a) <u>Cell extracts:</u>		
O—O	0°C + azide	296
X—X	37°C + azide	186
●—●	37°C - azide	158
b) <u>Incubation supernatants:</u>		
X—X	37°C + azide	106
●—●	37°C - azide	129

6.2.3.1 Ig complexes retained by labeled cells

In the case of cells kept at 0°C, the sedimentation pattern of their NP40 extracts (Fig. 6.4a) was essentially the same as that seen in Fig. 6.1a, except for the presence (40 % of total radioactivity) of dissociated 7S antibody.

For cells incubated at 37°C in the *absence* of azide, the sedimentation pattern of their extracts differed in several ways from that of cells kept at 0°C: (a) Almost no 21S complex was found. (b) The ratio of 11S complex/7S dissociated antibody was unchanged, although the total radioactivity in both fractions was less. (c) A significant proportion (35 %) of the total radioactivity was now in a 3S component. The proportion of this 3S material increased with time of incubation and by 3-4 h it was the major radioactive component in the cell extracts.

For cells incubated at 37°C in the *presence* of azide, the sedimentation pattern of their extracts was similar to that of cells incubated at 37°C without azide, except *almost no 3S component was present*. However, with prolonged incubation of cells (4-5 h) this component did slowly appear.

From these results, several conclusions may be made: (a) The 21S (IgM) complex rapidly disappeared from labeled cells at 37°C, but not at 0°C. Azide did not affect this process. (b) The 11S (IgM) complex slowly disappeared at 37°C, but not at 0°C. Antibody dissociated by NP40 (*i.e.* the 7S peak) also disappeared at a similar rate. Azide had no effect on either loss. (c) At 37°C, but not at 0°C, a significant proportion of the cell-associated radioactive complexes were degraded to a 3S component, this proportion increasing with time of incubation. Azide significantly inhibited the rate at which this phenomenon occurred.

6.2.3.2 Ig Complexes released by labeled cells at 37°C

The 37°C incubation supernatants gave similar sedimentation patterns whether or not they contained azide, except the medium lacking azide contained a trace of 2-3S material (Fig. 6.4b). A proportion (22 %) of antibody was uncomplexed (7S peak), indicating that some dissociation occurred at 37°C (neither sample was exposed to NP40). The

radioactivity in these supernatants was present as (a) dissociated antibody (22 %), (b) 11S complex (25 %) and (c) 21S complex (20-25 %), together with more aggregated material (20 %). The ratio of 21S/11S complexes in the 37°C supernatants was larger than in extracts of cells incubated at 0°C, indicating that the 21S complex eluted more rapidly than 11S. These results support those of section 6.2.3.1.

When intact cells or an NP40 cell extract were incubated at 37°C with a soluble complex formed between labeled anti- μ -chain IgG and sheep IgM, only the extract was capable of degrading the complex to material sedimenting at <7S (2.9 % degraded in 4 h/37°C by 10^8 cells/ml in DBSS/0.5 % NP40/15 mM NaN₃). Thus, the protease(s) responsible for this degradation were not available on the cell surface, but were released when the cells were dissolved. Furthermore, little 3S material formed from cell surface-bound label was found in the incubation supernatant lacking azide (above). These results indicate that this degradation probably occurred intracellularly, azide presumably preventing surface-bound material from being (actively) endocytosed.

6.2.4 Estimation of Ig present on the lymphocyte surface and in the medium during culture

The fate of (Ig/anti-Ig) complexes on the cell surface is not necessarily indicative of the behaviour of uncomplexed surface-Ig, nor does it indicate whether the Ig is a product of the cell or merely absorbed to it (cytophilic). This could be determined if cells were incubated at 37°C and the Ig present on their surface and in the medium estimated at different times. Lymphocytes (1×10^7 cells/ml) were incubated at 0°C or 37°C for periods up to 20 h. At various times, cells were removed, labeled with anti- γ or anti- μ -chain antibody and examined by autoradiography. The supernatants were also examined for their Ig content.

6.2.4.1 Ig at the cell surface

The results of autoradiographs of cells labeled after various periods of incubation are shown in Fig. 6.5. It is apparent that at 0 h most of the cells binding anti- γ -chain IgG were lightly labeled and that during incubation at 37°C

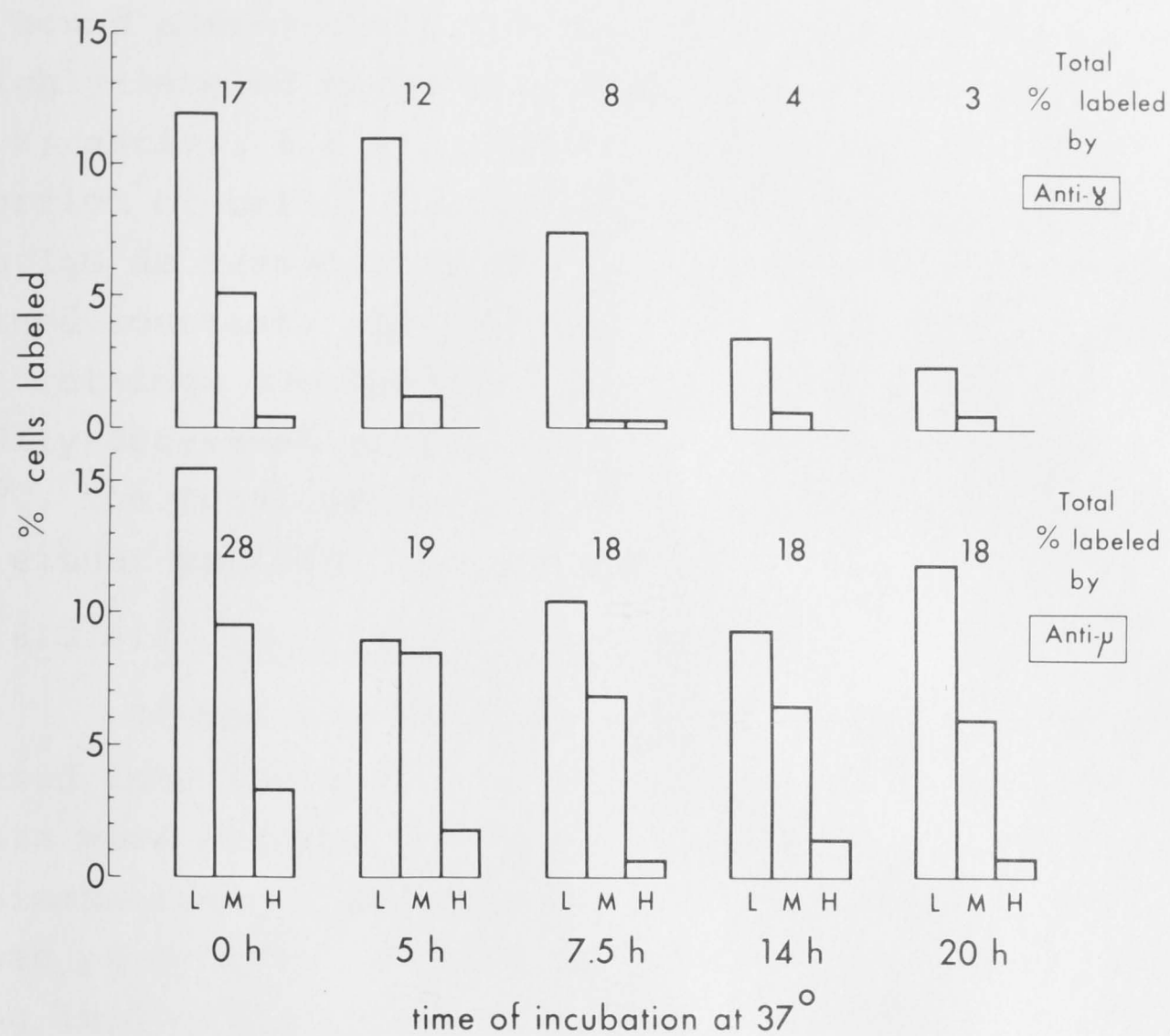


Figure 6.5: Incidence of lymphocytes binding ^{125}I -labeled anti-Ig antibodies after various periods of culture at 37°C .

Lymphocytes were incubated at 1×10^7 cells/ml in Eagle's medium plus 10 % FCS. After various periods, samples were removed, the cells sedimented (750xg, 5 min) and resuspended in DBSS/10 % NRS/15 mM NaN_3 at 0°C and labeled with either ^{125}I -labeled anti- γ or anti- μ chain antibody (250 ng/ml, 60 min, 0°C). The cells were then washed and smeared for autoradiography.

The figure shows the relative frequencies of intact cells which underlay different numbers of grains (11 to >50) in autoradiographs. Categories estimated in terms of grains/cell were: 11 - 25 (L); 26 - 50 (M) and >50 (H). The figures above each time point indicate the total percentage of cells labeling (L + M + H).

these slowly lost their ability to bind this antibody. At 0 h, there were more cells which bound anti- μ antibody (28 %) than bound anti- γ -chain (17 %). Furthermore, the proportions of highly-labeled cells were even more different (anti- μ , 12.8 %; anti- γ , 5.4 %). During incubation at 37°C, the total proportion of cells which retained the ability to bind anti- μ -chain IgG decreased from 28% to 19 % by 5 h and thereafter remained constant. In contrast, the total proportion of cells which retained the ability to bind anti- γ -chain IgG (17 %) steadily decreased so that by 20 h the proportion was only 3 %. At 0°C, the total proportion of cells retaining the ability to bind either antibody did not change.

6.2.4.2 Ig in the culture medium

It was now important to determine whether Ig was released into the medium by lymphocytes. Incubation supernatants were assayed for their content of IgG and IgM by radioimmunoassay. The results are shown in Fig. 6.6. At 0 h, IgG was present at 10 ng/ml and this level did not increase during incubation at 0°C. At 37°C, IgG increased steadily at 10 ng/ml/h for the first 8 h, then more rapidly at 25 ng/ml/h up to 20 h.

The estimation of sheep IgM 'secreted' into the medium was difficult owing to substantial interference by the medium itself. If the degree of this interference in the assay versus the control media was assumed to be similar, then it could be calculated that the increase in sheep IgM concentration in the medium between 5 and 14 h was of the order of 15 ng/ml/h. Because of the degree of interference and the low levels of secreted IgM, no attempt was made to distinguish 19S and 7S IgM in the medium.

6.2.5 Estimation of 19S IgM on the surface of lymphocytes after incubation at 0°C or 37°C

It was of interest to determine the nature of the IgM remaining on the cell surface after incubation of lymphocytes in culture medium. Thus cells were incubated at 0°C or 37°C for 5 h before being labeled with anti- μ -chain IgG. They were then dissolved and the extracts analyzed by sedimentation. It was found that the proportion of 21S complex was similar in

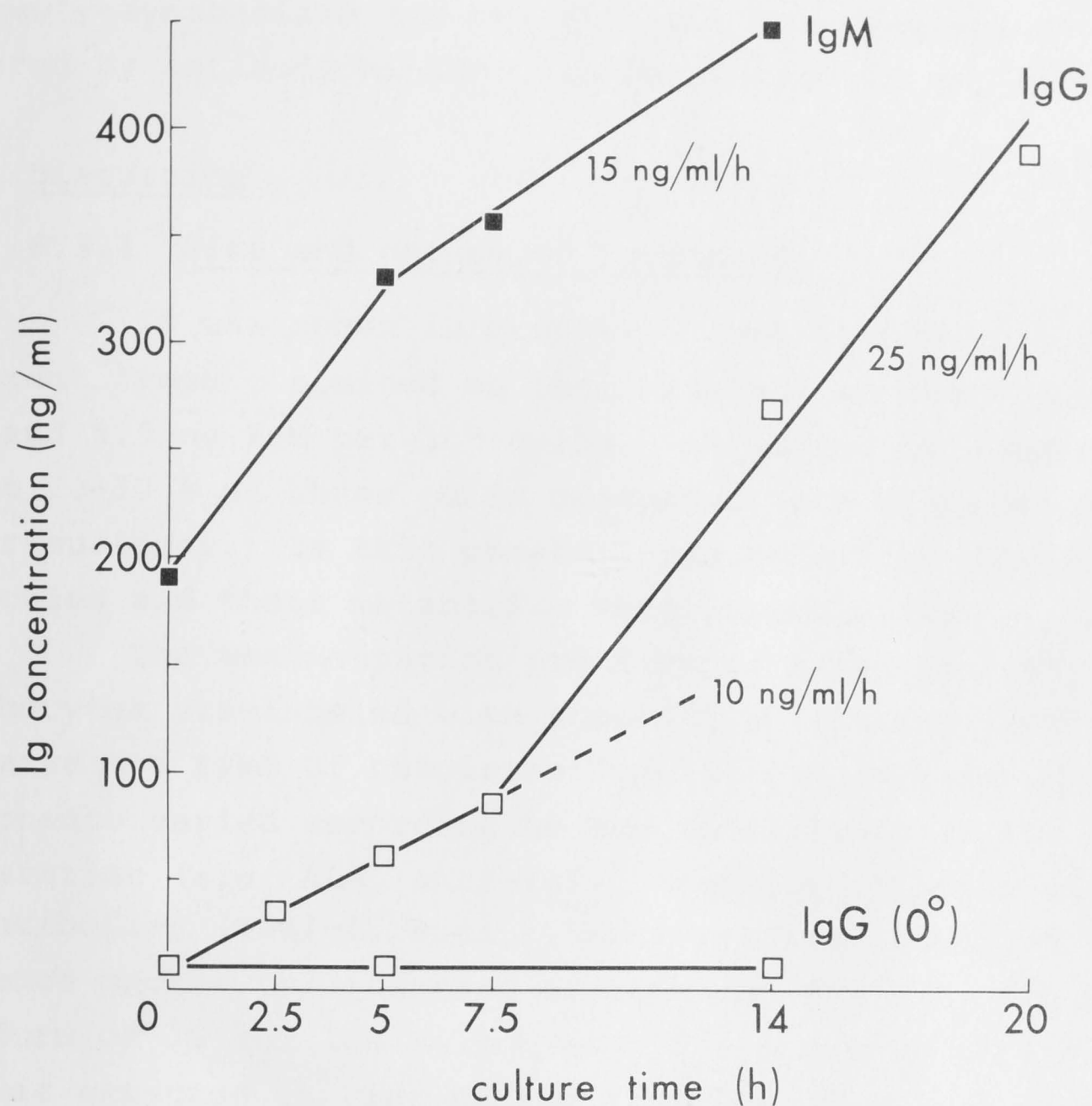


Figure 6.6: Release of Ig by sheep lymphocytes incubated in culture medium.

Lymphocytes (1×10^7 cells/ml) were incubated at 0° or 37°C in culture medium. At the indicated times, samples were removed and centrifuged ($750\times g$, 10 min) to remove cells. The supernatants were then tested by radioimmunoassay for their IgG and IgM content. The unexpected high 0 h estimate for IgM was presumably due to interference by the culture medium as similar values for IgM were obtained using the supernatants of cells incubated at 0°C .

each case (12-13 % of total radioactivity), indicating that 19S IgM may be either (a) released into the medium, but replaced by newly-synthesized IgM or (b) not released unless it is altered by antibody binding, in which case it may be cytophilic.

6.3 Discussion

6.3.1 Size and nature of surface-Ig

It was shown in Chapter 5 that lymphocytes from sheep efferent lymph contained on their surface an average of 1.2 ng IgG and 5.5 ng IgM per 10^6 cells. As judged by autoradiography, about 20-30 % of these cells possessed both classes of Ig on their surfaces. In this chapter, the molecular size of these molecules and their metabolism were investigated.

The sedimentation profiles of extracts from lymphocytes pre-labeled with specific antibodies indicated that the size and type of complexes formed with surface membrane components varied according to the specificity of the antibody preparation (e.g. ALG, anti-Ig). Reaction of cells with anti-Ig antibodies (anti-L, anti- γ , anti- μ -chain) revealed the presence on the cell surface of both IgG and IgM, the latter in the form of 7S and 19S molecules. The presence of IgG and 7S IgM was expected in view of the findings of others (Eskeland et al., 1971; Vitetta, Baur and Uhr, 1971; Marchalonis, Cone and Atwell, 1972). With one exception (Melchers and Andersson, 1973), no other investigators have reported the presence of significant amounts of 19S IgM on the cell surface. Yet 20-40 % of the total IgM on sheep lymphocytes was 19S. Its presence on these cells could reflect: (a) Cytophilic IgM from plasma, which is considered later. (b) The presence of a small number of activated antibody-secreting cells. This is unlikely because the source of lymphocytes (efferent lymph from nodes of unstimulated sheep) was chosen for its low content of antibody-secreting cells (Hall and Morris, 1962). To account for surface 19S IgM in the amounts detected, the number of such cells would need to be a readily-detectable proportion of the cells binding anti- μ -chain antibody (25 % of all lymphocytes). Moreover, if the 19S surface-IgM detected was because of the presence of antibody-secreting cells, it would presumably be more readily observed in spleen suspensions (Vitetta, Baur and Uhr, 1971;

Marchalonis, Cone and Atwell, 1972; Vitetta and Uhr, 1972a,b).

(c) The possibility that 19S IgM represents a functionally important component of the cell membrane will be considered in the general discussion (Chapter 8).

6.3.2 Loss and replacement of surface-Ig during incubation in vitro

To determine whether the surface-Igs are cytophilic or intrinsic to the cells, lymphocytes were incubated at 37°C for various periods before labeling as above. The results from these experiments are summarized in Table 6.2. These calculations together with results given earlier allow two main conclusions:

(a) IgG on most cells was shed without replacement so that a large proportion, but not all of the surface-IgG was probably cytophilic. However, loss of only surface-IgG (initially, 1.2 ng/10⁶ cells) cannot account for the total IgG released over 20 h (40 ng/10⁶ cells). Was IgG actively secreted from all the cells? Or was there a small proportion of very active antibody secreting cells? As a comparison, IgG from MOPC 21 myeloma cells and IgM from spleen cells of mice immunized with sheep red blood cells are released at rates of 6,300 ng/h/10⁶ cells (Melchers, 1970) and 3,800 ng/h/10⁶ plaque-forming cells (Hiramoto, Hamlin and McGhee, 1972) respectively. Thus, it would require only a very small proportion (0.04 %) of such rapid secretors in the lymphocyte population to account for the observed IgG release. Clearly, either or both situations may be occurring. Other studies have indicated that mouse and rabbit lymphocytes contain more intracellular than surface IgG (Marchalonis, Cone and Atwell, 1972; Pernis, Forni and Amante, 1971) and IgG is synthesized and secreted in greater amounts than IgM by mouse lymph node cells (Parkhouse, 1973).

(b) In contrast, a high proportion (70 %) of the cells originally binding anti- μ -chain antibody retained this ability after 20 h incubation at 37°C (Table 6.2). The major part of this 30 % decrease occurred during the first 5 h incubation, when the lymphocytes still possessed both 19S and 7S IgM in an unchanged ratio (1:2). Though it seems possible that some of the IgM may have been cytophilic, there was replacement of much of the surface IgM and this probably applies

Table 6.2

Loss and replacement of sheep lymphocyte surface-Ig

	<u>Initial surface-Ig^{a)}</u> (ng/10 ⁶ cells)	<u>Ig Released^{b)}</u> (ng/10 ⁶ cells/h)	<u>% Cells labeled^{c)}</u>		
			<u>0 h</u>	<u>5 h</u>	<u>20 h</u>
IgG	1.2 ± 0.5	1.0 - 2.5	17	12	3
IgM	5.5 ± 3.5	approx. 1.5	28	19	18

a) Estimated using intact cells as inhibitors in radioimmunoassay.

b) Ig released at 37°C by 10⁷ cells/ml, as estimated by radioimmunoassay (see Fig. 6.6).

c) Percentage of cells binding anti-γ or anti-μ chain antibody at 0°C, as judged by autoradiography, after incubation at 37°C for the period indicated. See Fig. 6.5.

to both 7S and 19S IgM. Because of the very small quantities involved, a final quantitative determination of the rate of synthesis or replacement of membrane-Ig must depend upon *in vitro* synthetic studies, such as has been done using mouse lymphocytes (Vitetta and Uhr, 1972a,b; Melchers and Andersson, 1973; Parkhouse, 1973) and human leukemic lymphocytes (Lerner, McConahey, Jansen and Dixon, 1972).

6.3.3 Fate of (anti-Ig/surface-Ig) complexes present on lymphocytes

The fate of (anti-Ig/surface-Ig) complexes present on the lymphocyte surface was studied during incubation of cells at 37°C, as this has not been reported in detail previously. Fig. 6.7 is an attempt to put into one diagram the various processes which were seen to occur, in the presence or absence of sodium azide, during a time period of 4 h. The following conclusions can be derived:

- (a) *Dissociation of complexes.* During the first 30-45 min. at 37°C, about 15 % of the 7S antibody which was complexed to cell surface-Ig at 0°C became dissociated and was 'lost' into the culture medium. This dissociation did not continue after 1 h incubation. Others have also observed this phenomenon (Wilson, Nossal and Lewis, 1972).
- (b) *Loss of complexes from cells.* Complexed antibody (radioactive material >7S) was also lost from cells and although this loss continued after 1-2 h, it had almost stopped by 4 h.
- (c) *Degradation of complexes retained by cells.* Radioactive material (complexed antibody) retained by cells after 1-2 h incubation was steadily degraded to material sedimenting at about 3S. By 3-4 h, there was as much degraded as undegraded material. Degraded material was not released immediately into the culture medium, but accumulated within the cells.
- (d) *Effect of azide.* The presence of azide (15 mM) did not alter the dissociation or loss of complexes from lymphocytes into the medium. In contrast, the drug markedly inhibited the rate at which radioactive material retained by the cells was degraded. This azide concentration (15 mM) has been shown elsewhere (Taylor *et al.*, 1971; Loor, Forni and Amante, 1972) to inhibit energy-requiring cellular processes. Others (Wilson, Nossal and Lewis, 1972; Cone, Marchalonis and Rolley, 1971) have

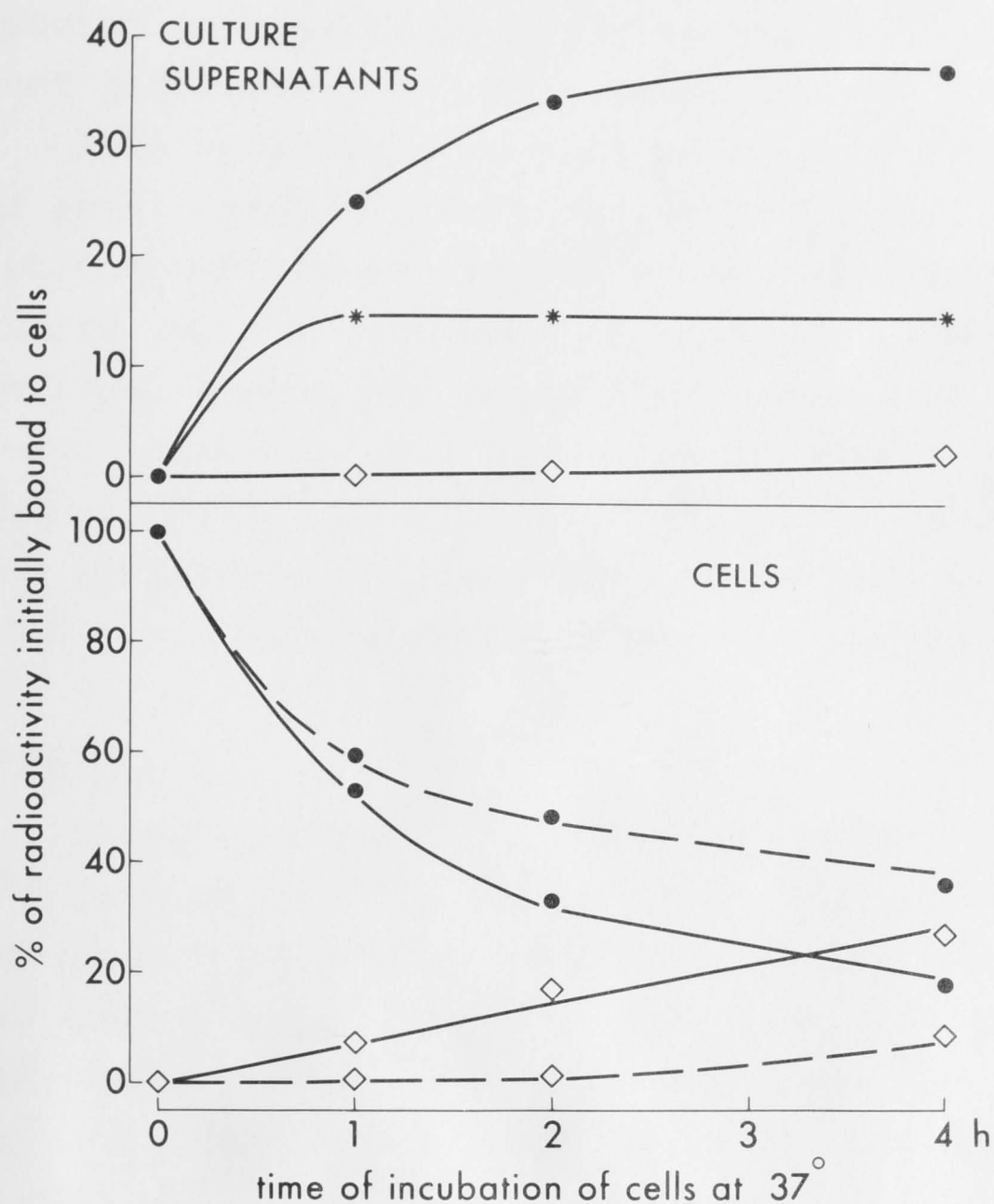


Figure 6.7: Fate of radioactive anti-Ig antibody bound to sheep lymphocytes.

The figure depicts the disappearance of radioactive complexes (●) from cells, prelabeled with anti-L, anti- γ or anti- μ chain antibody, and incubated at 37°C in the presence (---) or absence (—) of 15 mM NaN₃. It also shows the appearance in the culture medium of dissociated (*), complexed (●) and degraded (◇) antibody and in the cells, of degraded antibody (◇). Values are only approximate. Radioactive material was analyzed by sedimentation centrifugation of culture supernatants and of NP40 cell extracts (as per Fig. 6.4). Degraded, dissociated and complexed antibody refers to radioactive material sedimenting at <7S, 7S and >7S respectively. Loss of material into the supernatants was unaffected by 15 mM azide.

used 10 times this concentration, at which level it has (a) been reported to kill cells (Vitetta and Uhr, 1972b) and (b) may affect processes other than respiration.

This work confirms earlier conclusions (Marchalonis, Cone and Atwell, 1971; Vitetta and Uhr, 1972a,b) that there is metabolic turnover (replacement) of cell surface-Ig, particularly IgM. It provides no evidence to support the claim (Wilson, Nossal and Lewis, 1972) that loss of (surface-Ig/anti-Ig) complexes is a metabolic process and this, together with other findings (Vitetta and Uhr, 1972b), strongly suggests that much surface-Ig is lost from lymphocytes during incubation via a non-metabolic process (e.g. diffusion).

6.4 Summary

Sheep lymphocytes possessing surface-Ig were labeled with ^{125}I -labeled anti-Ig antibodies. The radioactive complexes thus formed were dissolved from the cell membrane by treatment with the detergent Nonidet P-40 and their molecular sizes estimated by sedimentation centrifugation. IgG and IgM (20-40 % as 19S IgM) were shown to be present on the cell surface.

Cells labeled with anti-Ig antibodies and incubated at 37°C lost 30-40 % of their radioactivity within 30-45 min., after which time the rate of loss decreased. By 3-4 h, approximately 50 % had been released and loss had almost stopped. Label associated with surface-bound ALG was also lost rapidly (20-30 % in 30 min.), but unlike anti-Ig antibodies, no further loss occurred. Anti-Ig complexes retained by labelled cells during incubation at 37°C were steadily degraded to a 3-4S component. Sodium azide (15 mM) significantly reduced the rate at which this degradation occurred (e.g. inhibition relative to degradation in the absence of azide was 95 % after 2 h and 67 % after 4 h incubation, respectively).

When unlabeled lymphocytes were incubated at 37°C for up to 20 h, and then reacted with labeled anti-Ig antibody, the following was observed. The proportions of lymphocytes able to bind anti- γ or anti- μ -chain antibody (as judged by autoradiography) decreased from 17 % to 3 % and 28 %

to 18 %, respectively, during 20 h incubation. From measurements of Ig released during incubation, it was concluded that most of the IgG initially on cells binding anti- γ -chain antibody was probably cytophilic. IgM initially on the surface of cells binding anti- μ -chain antibody seemed to be released and replaced by the cells, although a small proportion may have been cytophilic.

- 7.1 Introduction
- 7.2 Experimental results
- 7.3 Discussion
- 7.4 Summary

7.1 Introduction

The receptor for antigen on unstimulated lymphocytes, particularly B cells, is thought to be immunoglobulin, because the evidence is so strongly in its favour that no alternative candidates have been proposed (see Chapter 1). This evidence is circumstantial however, and is based mainly on the ability of anti-Ig sera to inhibit the binding of antigens to ABC and to inhibit functional inactivation of lymphocytes by radiolabeled antigens. Such results do not distinguish between the possibilities that the receptor is Ig or that Ig is so closely adjacent to the "true" receptor that anti-Ig antibodies which bind to the Ig sterically interfere with the interaction between the "true" receptor and antigen (Fig 7.1).

What evidence is needed to show unequivocally that Ig is the functional receptor for antigen? We would be one step closer to showing this if it could be demonstrated that unless antigen bound to Ig, the cell was not stimulated. Thus we need to demonstrate that under conditions where cells are stimulated, (antigen/Ig) complexes can be isolated from the cell and identified; under conditions where stimulation does not occur (but not by inactivation or tolerance), such complexes cannot be isolated.

The approach chosen to implement this idea was based on: (a) the demonstration by autoradiography of cells (ABC) which *specifically* bind radiolabeled antigen; (b) the ability of radioactive antigen to "suicide" immunocompetent cells; and (c) the ability of anti-Ig sera to prevent the binding of antigen to ABC and to prevent "suicide" (Chapter 1). Thus, the (antigen/receptor) complexes which are involved in specific stimulation of lymphocytes should be found in cells labeled with antigen in the *absence*, but *not* in cells labeled in the *presence*, of anti-Ig sera. This difference represents a potential means for isolating and identifying the immunologically-important (antigen/receptor) complexes and thus the specific

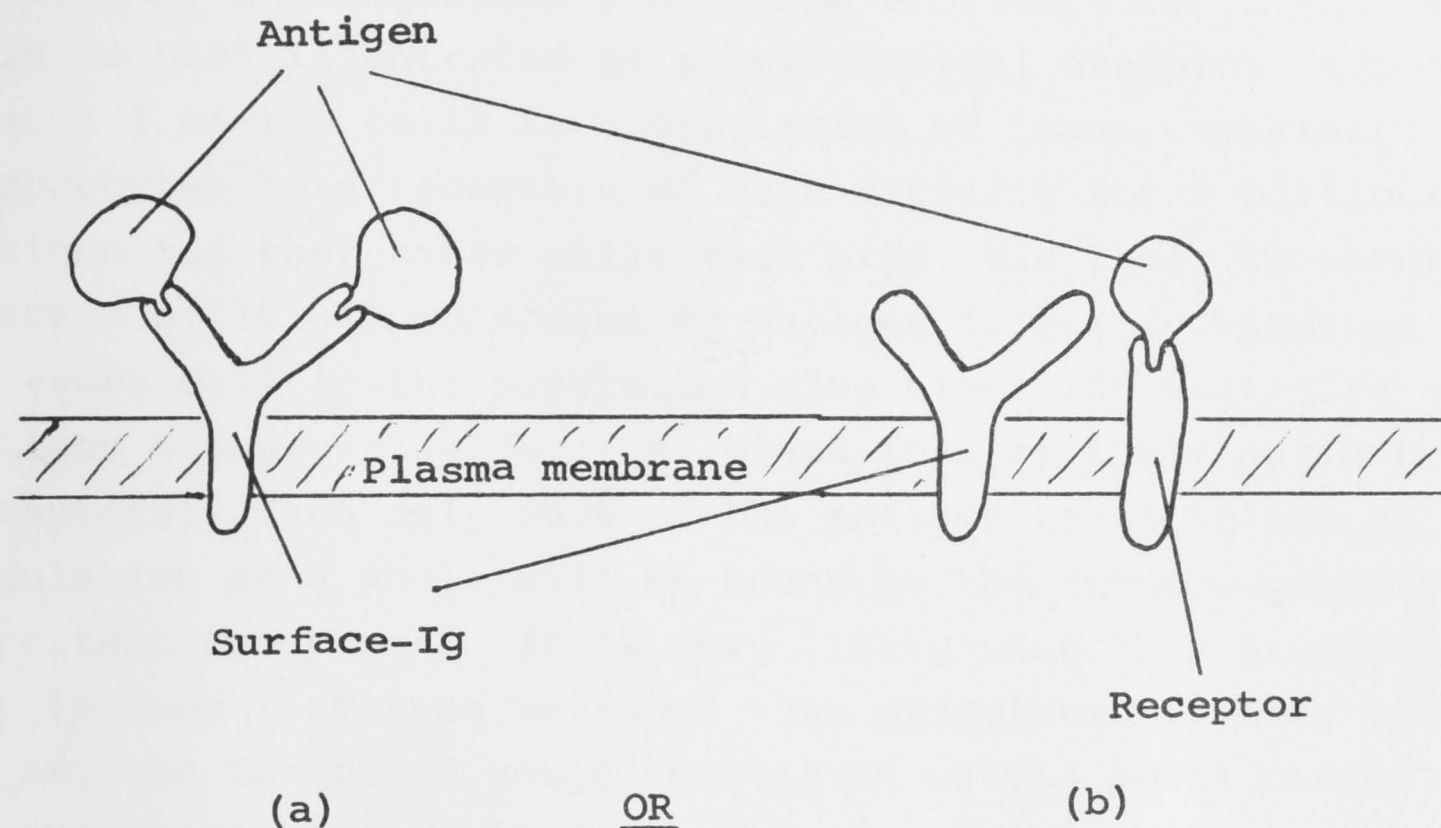


Figure 7.1: Possible relationships of surface-Ig to the receptor for antigen

(a) Surface-Ig = true receptor

(b) Surface-Ig \neq true receptor, but is situated adjacent to it so that anti-Ig antibodies binding to the Ig sterically hinder the accessibility of the receptor to antigen.

receptors present on those cells which bind and become stimulated by a particular antigen.

It was realized, however, that certain difficulties might have to be overcome in pursuing this approach. For instance, the heterogeneity of the receptor molecules in a population of lymphocytes has so far prevented the characterization of a homogeneous population of receptors in this way. This is best illustrated by a hypothetical example: Suppose that 1 % of the cells in a population of immunocompetent lymphocytes have receptors of high affinity for a particular antigen and that these cells each bind, via their receptors, an average of 10,000 molecules of antigen (*specific binding*). Now if every cell in the population also binds 100 molecules of the antigen *nonspecifically* (i.e. other than by their antigen-receptors), then only 50 % of the antigen bound to the cell population as a whole will be bound to the immunologically-important receptors. It is very likely that this proportion may in many instances be lower than calculated in this example. If so, the receptors would constitute only a small proportion of the total antigen-binding capacity of the cells and may be only one of a number of cell surface components with which antigen becomes associated.

In addition to the problem of nonspecific binding, the (presumed) noncovalent interaction of antigen with cell receptors necessitates the avoidance of conditions which might dissociate them during their isolation. This limits the methods which can be used to either disrupt the cells or identify antigen-associated cellular components. Furthermore, if more than one class of compound is found associated with antigen, the importance of each in the stimulation of lymphocytes by antigen must then be determined if one is to be unequivocally identified as the true (immunological) receptor for antigen.

This chapter describes preliminary experiments designed to establish the feasibility of an approach of this kind.

7.2 Experimental results

7.2.1 Ability of anti-IgM serum to inhibit net binding of antigens to mouse or sheep lymphoid cells

The extent to which an antigen binds nonspecifically to lymphocytes, *i.e.* other than by the antigen-specific-receptors, may reasonably be expected to depend on the nature of the antigen (*i.e.* some antigens should be more cytophilic than others). In order to determine whether an antigen could be found which binds to lymphocytes mainly through their surface receptors, the *net* binding of a number of antigens to mouse lymph node, spleen, or sheep lymph cells pretreated with normal rabbit serum or with anti-IgM serum was determined. The results are shown in Table 7.1.

Firstly, it is clear that the total amount of antigen which bound to the cell populations was not the same for each of the antigens tested, and that different cell preparations bound different quantities of the same antigen. Secondly, the effect of pretreating the cells with anti-IgM serum was variable: In some cases antigen binding was enhanced; in others the binding was reduced. This result may be due to the "coating" of IgM⁺ cells by anti-IgM antibodies which may block receptor-specific binding of antigen to a small proportion of cells (ABC), but which may also increase the non-receptor binding of cytophilic antigens to the majority of cells.

Haemocyanin (HCY) was the only antigen whose binding was significantly reduced by pretreating cells with anti-IgM serum (the average inhibition in its binding to mouse spleen cells was 83 %). The binding of *Salmonella* flagellar proteins (monomeric, and polymeric) and of BSA (DNP)₇ was reduced in some instances, but never by more than 33 %, and enhanced binding was observed in several experiments. These results may be compared with the marked degree (> 80 %) to which anti-Ig sera inhibit the binding of these antigens to individual ABC as determined autoradiographically (Warner, Byrt and Ada, 1970; Dwyer and Mackay, 1972). Each antigen, except HCY, must therefore have been bound to lymphocytes and other cells via anti-Ig-insensitive sites in greater *net* amounts than to ABC via anti-Ig-sensitive sites. On this basis, HCY seems to be a suitable antigen for use in isolating anti-Ig-sensitive, HCY-specific lymphocyte receptors. Furthermore, ¹²⁵I-labeled HCY is able to specifically reduce the ability of mouse lymphoid cells to transfer antibody responsiveness to lethally-

Table 7.1

Ability of anti-IgM serum to inhibit *net* binding of antigens
to populations of mouse or sheep lymphoid cells

Antigen	Percentage of ^{125}I -labeled antigen bound to cells ^{a)}								
	Mouse mesenteric lymph node cells			Mouse spleen cells			Sheep efferent popliteal lymph cells		
	NRS	Anti-mouse-IgM	Inhibition (%)	NRS	Anti-mouse-IgM	Inhibition (%)	NRS	Anti-sheep-IgM	Inhibition (%)
BSA (DNP) ₇ ^{b)}	0.034	0.061	(-44)	-	-	-	0.042	0.037	(12)
	0.020	0.025	(-20)	-	-	-	-	-	-
MON 870	0.35	0.37	(-5)	-	-	-	0.36	0.29	(19)
POL 870	0.41	0.35	(15)	0.085	0.071	(15)	0.49	0.71	(-31)
	0.69	0.77	(-10)						
POL 1338	0.062	0.055	(11)	0.93	0.80	(14)	-	-	-
	0.409	0.272	(33)						
HCY	0.342	0.089	(74)	0.388	0.064	(84)	-	-	-
				0.361	0.066	(82)			

a) Cells (10^8 cells/ml) were incubated at 0°C for periods of 2-6 h with normal rabbit serum (NRS) or anti-IgM serum at final dilutions of $\leq 1/5$. The serum was then removed and the cells were incubated at 10^8 cells/ml for 1 h at 0°C in DBSS containing 10 % FCS, 15 mM NaN_3 , and ^{125}I -labeled antigen at concentrations between 350 and 1250 ng/ml. The cells were then washed at $0-4^\circ\text{C}$ with DBSS/10 % FCS/15 mM NaN_3 (DFN) and the proportion of antigen bound to the cells determined by bulk scintillation counting. Sera were diluted with DFN.

b) Cells were incubated in FCS, which should contain a large excess of unlabeled BSA.

irradiated recipients (Ada, personal communication) and this antigen could therefore be used to test the functional significance of a receptor which might be found associated with it.

7.2.2 Ability of various anti-mouse-Ig sera to inhibit the binding of ^{125}I -labeled haemocyanin to mouse spleen cells

Titration curves showing the capacity of rabbit anti-mouse-Ig sera to inhibit the binding of labeled HCY to mouse spleen cells are given in Fig 7.2. Pretreatment of cells with either anti- γ -chain or anti-IgM serum at dilutions of $\frac{1}{2}$ to $\frac{1}{50}$ resulted in a 60 % reduction in the amount of HCY bound to the cells, compared to that bound to cells pretreated with NRS. This was therefore the maximal inhibition which could be caused by either serum. Anti- μ -chain serum caused a 40 % reduction in binding at a dilution of $\frac{1}{2}$ or $\frac{1}{5}$, but was less effective at higher dilutions.

The degree of inhibition effected by anti- γ -chain serum was unexpected in view of the usually poor ability of this serum to block the binding of antigen to ABC as detected by autoradiography (Warner, Byrt and Ada, 1970). The inhibition observed may have been due to anti-L-chain antibody present in the serum, but the effectiveness of the serum at high dilution, together with its poor cross-reactivity with IgM in radio-immunoassay (Fig 7.2) make this seem unlikely. Nevertheless, the possibility that anti-L-chain antibody at very low concentrations is more effective than anti-heavy-chain antibodies in blocking the receptors cannot at present be excluded. Unfortunately no anti-L-chain serum was available when these experiments were done, and there was insufficient time to investigate by autoradiography the ability of these sera to inhibit binding of radiolabeled HCY to ABC.

7.2.3 Net binding of haemocyanin to mouse spleen cells. The importance of haemocyanin concentration and period of exposure to labeled haemocyanin

It is clear from Fig 7.3 that varying the length of the period for which cells were incubated with labeled HCY had

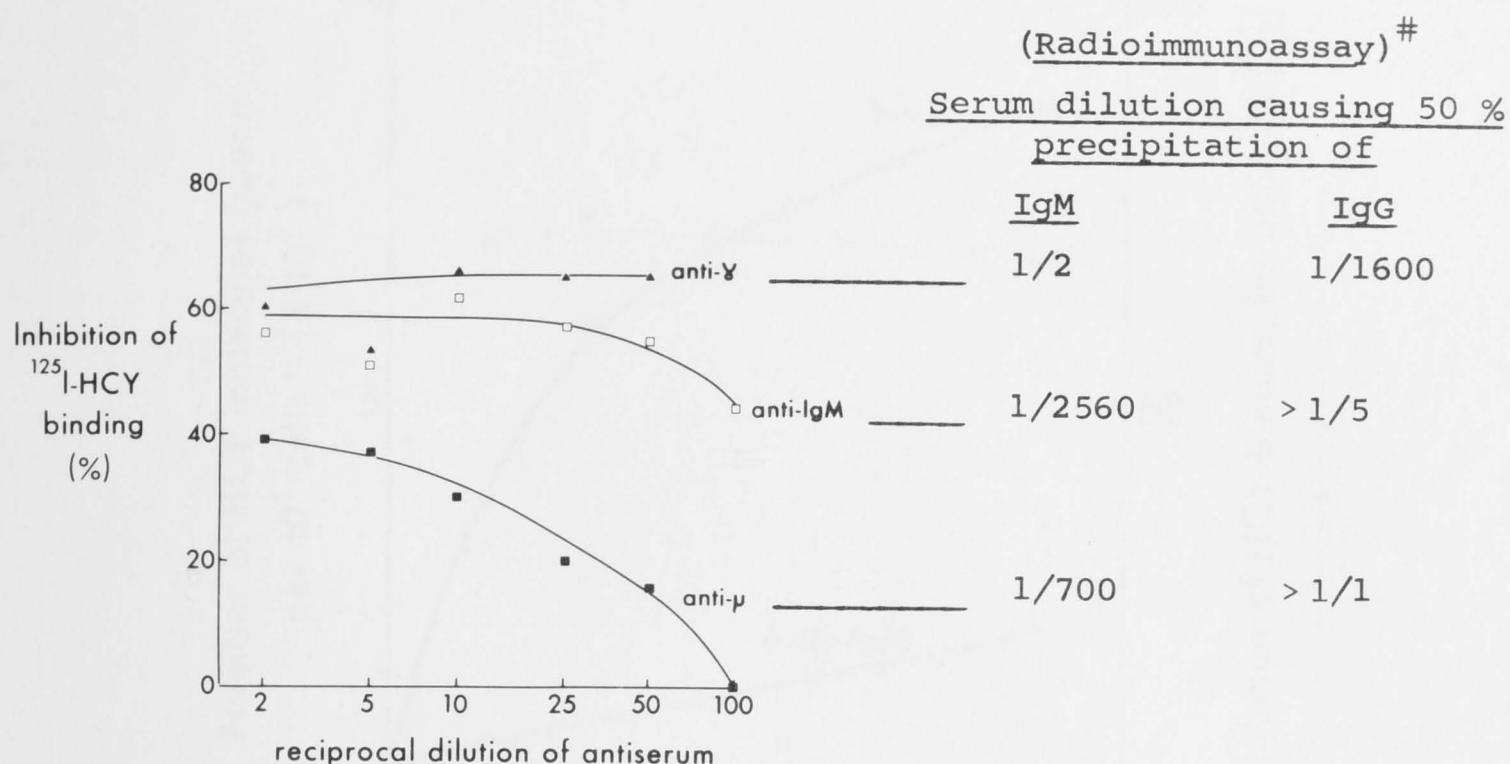


Figure 7.2: Net binding of ^{125}I -labeled haemocyanin to mouse spleen cells preincubated with anti-mouse-Ig sera.

The spleen cells from 4 CBA mice were pooled, washed in DFN* and divided into 24 equal fractions. The cells were sedimented and resuspended to 5×10^7 cells/ml in NRS, anti- γ -chain, anti- μ -chain, or anti-IgM serum diluted 1 in 2, 5, 10, 25, 50, or 100, with DFN. After incubation for 2 h at 0°C , the cells were sedimented, resuspended to 5×10^7 cells/ml in DFN containing labeled HCY at 220 ng/ml, and incubated at 0°C for 1 h. The cells were then washed at $0-4^\circ\text{C}$ in DFN and assayed for radioactivity by bulk scintillation counting.

The results are plotted as the percentage reduction in binding caused by each antiserum compared to the binding by cells pretreated with NRS at corresponding dilutions.

*DFN: DBSS containing 10 % FCS and 15 mM NaN_3 .

Dilution of each antiserum required to cause 50 % precipitation of 10 ng of either IgM or IgG by radioimmunoassay.

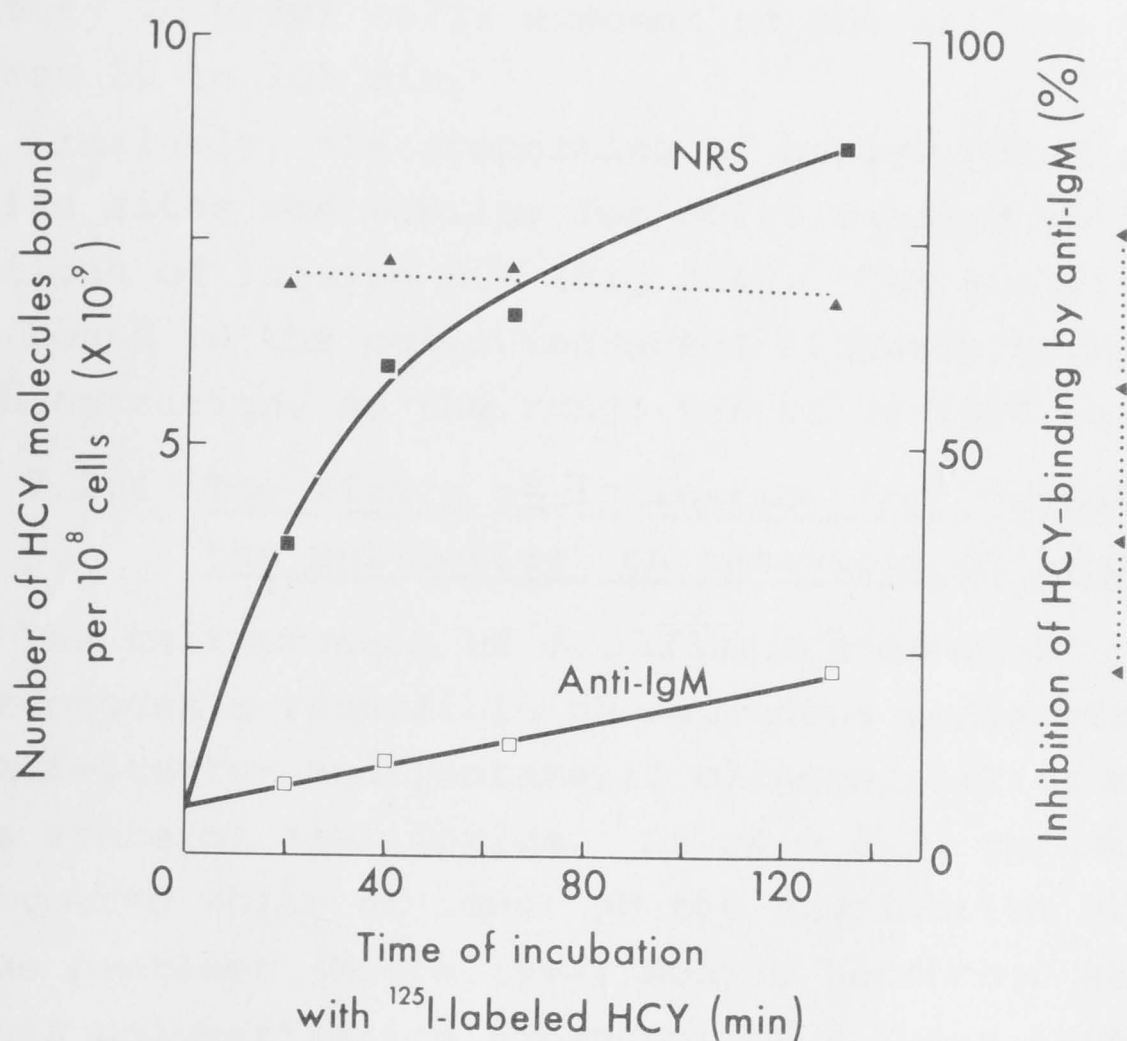


Figure 7.3: Time-course of the binding of ^{125}I -labeled haemocyanin to mouse spleen cells

The spleen cells from 5 CBA mice were pooled and washed in DFN*. One half of the cells were incubated in 25 % NRS, the other in 25 % anti-mouse-IgM serum, at 1×10^8 cells/ml for 90 min at 0°C . Each suspension was then centrifuged and the cells resuspended to 1×10^8 cells/ml in DFN at 0°C before the addition of ^{125}I -labeled HCY to 370 ng/ml. Samples (200 μl) were removed from each suspension at the indicated times. The cells were washed in DFN at $0-4^\circ\text{C}$ and then assayed for radioactivity. The results are plotted as the number of molecules of HCY bound per 10^8 cells, based on a molecular weight for HCY of 450,000 daltons.

*DFN: DBSS containing 10 % FCS and 15 mM NaN_3 .

little effect on the proportion of cell-associated HCY which was bound to anti-Ig-sensitive sites. The antigen bound to the cells most rapidly during the first 40 min. at 0°C, but the proportion bound to anti-Ig-sensitive sites was constant at approximately 70 % for cells exposed to the antigen for periods ranging from 20 to 135 min.

Similarly, the proportion of antigen binding to anti-Ig-sensitive sites was similar for cells exposed to different concentrations of labeled HCY (Fig 7.4). The number of HCY molecules bound to the cells increased linearly with respect to HCY concentration, in the range tested (0-1000 ng/ml).

7.2.4 The effect of iodination and oxidation on the polymerization behaviour of haemocyanin

The unit protein of *J. lalandii* haemocyanin (MW: 86,000) undergoes a reversible pH-dependent polymerization to form di-, tri-, tetra- and pentameric oligomers which normally exist in a state of equilibrium. At pH > 8.7, the monomeric form is favoured while at lower pH the equilibrium shifts towards the pentamer (Moore, 1967; Moore, Henderson and Nichol, 1968). This polymerization behaviour of HCY may interfere with the isolation and characterization of (HCY/receptor) complexes from lymphocytes labeled with HCY. As it would be preferable (for this purpose) to use a nonaggregating antigen, the effect of oxidation on the ability of HCY to polymerize was determined. This treatment was chosen because: (a) HCY may be less antigenic at pH > 8.7 than at lower pH; (b) The use of pH > 8.7 may cause dissociation of the (HCY/receptor) complex; and (c) Mild oxidation of *Salmonella* flagellin (the monomeric protein of flagella) is known to prevent its polymerization, but does not significantly alter its antigenic properties (Parish and Stanley, 1972).

To determine whether iodination/oxidation of HCY could similarly prevent its polymerization, aliquots of HCY which had been depolymerized at pH 9.1 were exposed at pH 9.1 and in the presence of 1×10^{-5} M iodide to chloramine-T at a number of different concentrations. It can be seen (Fig 7.5) that the efficiency of iodination of the protein increased as the chloramine-T concentration was raised, reaching a maximum of 75-80 % at 5×10^{-4} M chloramine-T. To determine the ability of

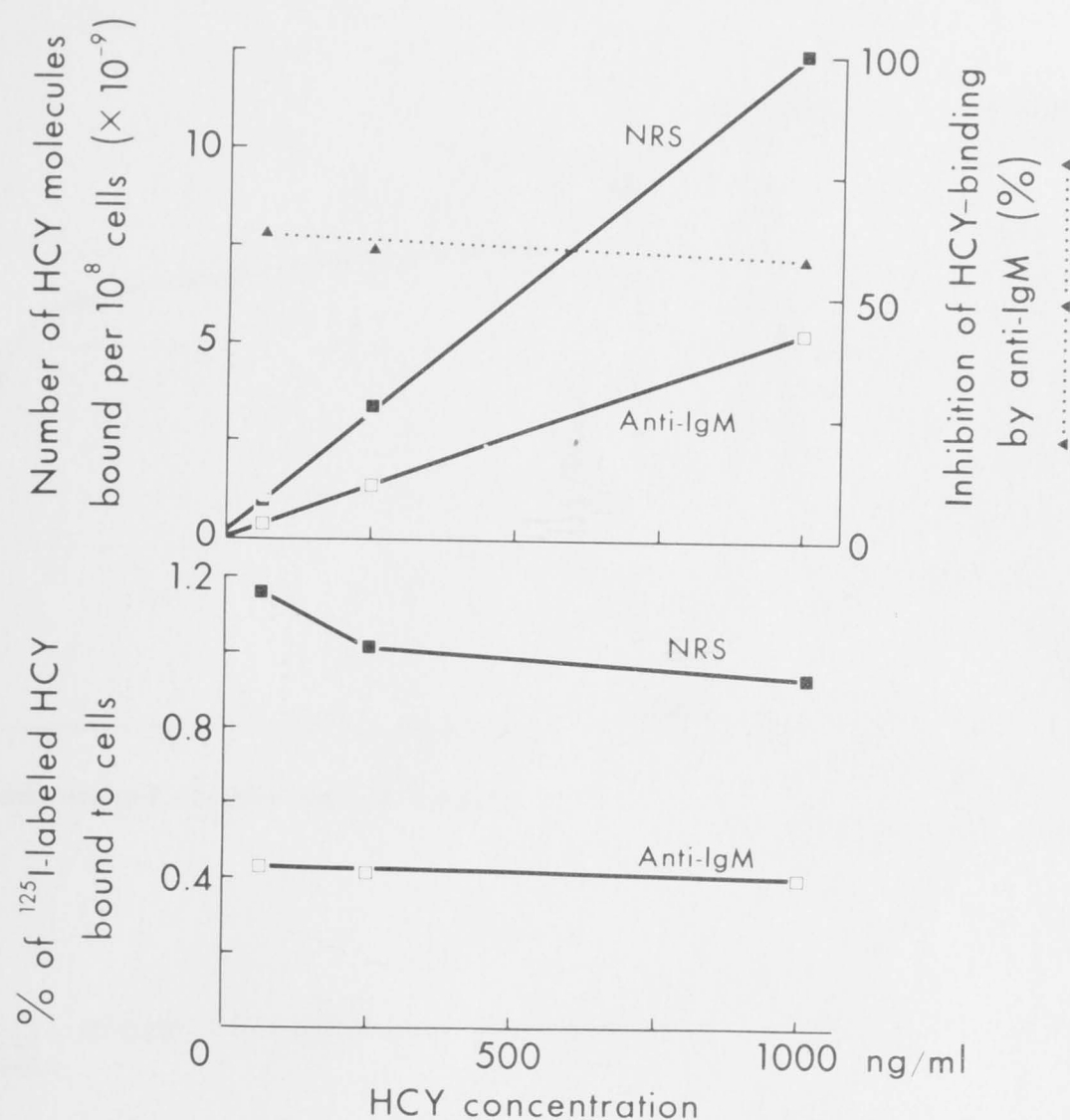


Figure 7.4: Binding of labeled haemocyanin to mouse spleen cells. Relationship to haemocyanin concentration

The pooled spleen cells of 6 CBA mice were washed in DFN* at $0-4^{\circ}\text{C}$. One half were incubated with 50 % NRS, the other with 50 % anti-IgM serum, at 1×10^8 cells/ml for 9 h at 0°C . Each suspension was then divided into 3 equal portions, centrifuged, and the cells resuspended in DFN at 1×10^8 cells/ml and incubated (1 h, 0°C) with labeled HCY at 62.5, 250, or 1000 ng/ml. The cells were then washed and assayed for bulk radioactivity. Results are expressed as Molecules of HCY bound per 10^8 cells (assuming a MW of 450,000 daltons) (TOP) or % of available HCY which bound to the cells (BOTTOM).

*DFN: DBSS containing 10 % FCS and 15 mM NaN_3 .

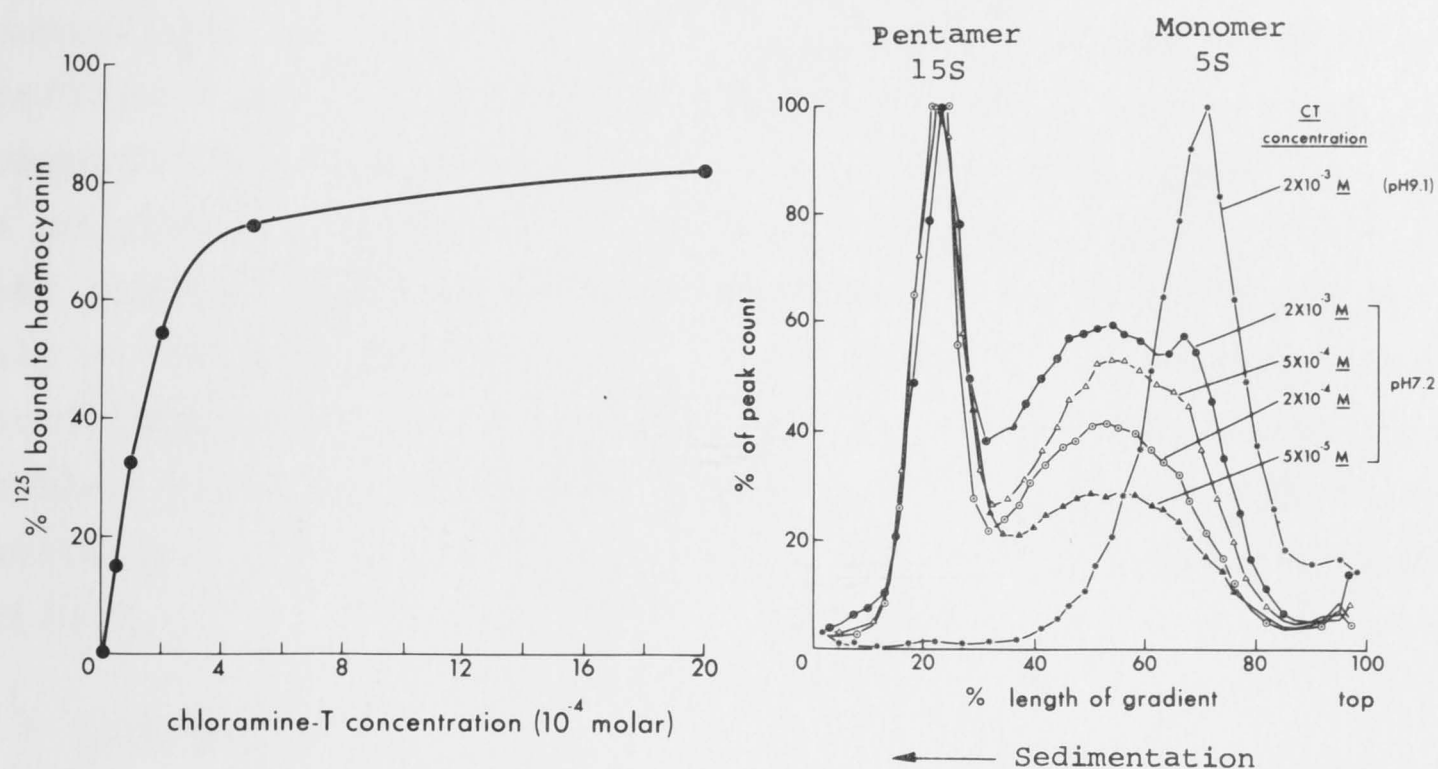


Figure 7.5: Effect of iodination and oxidation on the polymerization of haemocyanin

Depolymerized* haemocyanin ($12.5 \text{ mg/ml} = 1.5 \times 10^{-4} \text{ M}^\#$) was incubated at 25°C with radioactive iodide ($1 \times 10^{-5} \text{ M}$, $4 \text{ } \mu\text{Ci/ml}$) and chloramine-T at each of the indicated concentrations, in 0.1 M Tris-HCl , pH 9.1. After a 15 min reaction, $\text{Na}_2\text{S}_2\text{O}_5$ was added to a 2-fold molar excess with respect to chloramine-T.

*HCY (25 mg/ml) was depolymerized by incubation in 0.1 M Tris-HCl , pH 9.1 for 12 h at 4°C , then 6 h at 25°C .

$^\#$ Assuming a molecular weight of 86,000 (Moore, 1967; Moore, Henderson and Nichol, 1968).

LEFT: Efficiency of iodination.

Samples ($50 \text{ } \mu\text{l}$) of each reaction mixture were diluted with 0.5 ml of 0.1 M NaI . Their radioactivity was measured before and after dialysis against 500 ml of PBS1 (12 h , 25°C).

RIGHT: Repolymerization of iodinated haemocyanin

The pH of a $100 \text{ } \mu\text{l}$ aliquot of each reaction mixture was adjusted to 7.0 by the addition of $20 \text{ } \mu\text{l}$ of 0.2 M sodium acetate, pH 4.5. Each sample was then dialysed for 22 h at 25°C against 2 changes of $50 \text{ mM NaCl}/50 \text{ mM Tris-HCl}$, pH 7.2/ 15 mM NaN_3 (500 ml) and then centrifuged on a 10–30 % (w/v) sucrose gradient containing $0.5 \text{ } \%$ NP40/ $5 \text{ mM EDTA}/50 \text{ mM NaCl}/50 \text{ mM Tris-HCl}$, pH 7.2 (SW50.1 rotor; $34,000 \text{ rpm}$, 17 h , 10°C). As a control, an unneutralized sample of HCY which had been reacted with $2 \times 10^{-3} \text{ M}$ chloramine-T was centrifuged over a similar gradient, but at pH 9.1. All fractions were tested for radioactivity.

the HCY to repolymerize, each iodinated sample was neutralized and dialyzed for 22 h against a pH 7.2 buffer. HCY achieves equilibrium between its different forms in > 10 h at 20°C (Moore, 1967). After dialysis, the extent to which the HCY in each sample had polymerized was estimated by sedimentation centrifugation (Fig 7.5). It is clear from the sedimentation profiles that HCY which had been exposed to increasing concentrations of chloramine-T was correspondingly less able to polymerize normally at pH 7.2. However, this effect was at best partial and even after treatment with $2 \times 10^{-3}\text{M}$ chloramine-T, only a small proportion ($\leq 27\%$) of HCY remained in monomeric form. Such treatment is therefore unlikely to result in a stable, nonassociating derivative of HCY more useful than the native protein for studies on the lymphocyte receptor for antigen.

7.3 Discussion

The experiments described in this chapter represent an approach to the identification of antigen-receptors of lymphocytes which differs from most in that it aims to identify the receptors on the basis of their ability to bind antigen. It involves measuring the *net* antigen-binding capacity of lymphocyte populations, in contrast to ABC assays which measure the antigen-binding capacity of individual cells within a population (Chapter 1). Revoltella *et al.* have also measured *net* binding of antigen, but only for the purpose of enumerating the total number of receptors present on a *population* of cells which bind a given antigenic determinant (Revoltella, Martinello and Osler, 1973a,b). This was estimated by measuring the amount of antigen which bound to spleen cells from mice immune to the antigen compared to that which bound to cells from mice immune to unrelated antigens. These workers did not investigate the ability of anti-Ig sera to inhibit antigen binding by the cells, and gave no mention to the possibility of receptor isolation using antigen-binding as a means of identification.

The use of anti-Ig sera as a means of estimating receptor-specific binding of antigen was based on the ability of these reagents to inhibit the binding of antigen to antigen-binding (and immunocompetent) lymphocytes (Chapter 1). Any decrease in *net* binding of antigen resulting from pre-exposure

of cells to anti-Ig sera was therefore thought likely to be due to a decrease mainly of receptor-specific binding. It was considered essential to the feasibility of this type of study that *some* inhibition of *net* antigen-binding be observed after anti-Ig treatment of cells, as:

- (a) A *measurable* fraction of cell-associated antigen must be bound to the receptors if the latter are to be isolated and characterized as a result of their association with antigen,
- (b) Preferential blocking of the receptors results in differences in the type and number of antigen-labeled components which are isolated from "normal" and "blocked" cells and thus enables the (anti-Ig-sensitive) receptors to be distinguished from other nonspecifically-labeled material.
- (c) Preferential blocking might provide a means for demonstrating *functional* importance of the component(s) whose association with antigen is prevented. Thus, the association of radioactive antigen with certain cellular components might be shown necessary for the "suicide" of cells which are immunocompetent for that antigen.

The ability of anti-Ig sera to consistently and significantly inhibit the net binding of HCY to mouse lymphoid cells suggests that only a small proportion of all cell-bound HCY is not bound via HCY-specific antigen-receptors. In contrast, the binding of other antigens was much less affected by anti-Ig treatment. The consistently high ratio of specific to nonspecific binding of HCY may occur if HCY is recognized and specifically bound by a greater proportion of ABC than are many other antigens. HCY may also or alternatively be less "sticky" or cytophilic than other antigens. This characteristic seems to be independent of antigen-dose and time of exposure of cells to the antigen, at least in the range tested.

It is worthwhile considering the *net* amount of HCY bound to the cell populations in terms of molecules bound per cell. In Fig 7.4, a total of 1×10^{10} molecules of pentameric HCY was bound per 10^8 cells, at approximately 800 ng HCY/ml; this is equivalent to 1×10^4 molecules/100 cells. Thus, if 0.1 % of the cells accounted for the observed *net* binding, then these ABC would each have bound 100,000 molecules of HCY. This figure is very similar to the total number of surface-Ig

molecules present on Ig⁺ (B) lymphocytes (Rabellino *et al.*, 1971; Grey, Colon, Campbell and Rabellino, 1972; Unanue *et al.*, 1971; Chapter 5 of this thesis). The doses of HCY used here were insufficient to saturate all binding sites on the cells, and therefore the maximum binding capacity is unknown. However, it is possible that > 0.1 % of the cells bound significant amounts of HCY specifically, and thus the observed levels of *net* HCY binding are not inconsistent with the idea that most of the antigen is bound to a small number of HCY-specific ABC.

These experiments, although only preliminary, indicate the feasibility of a biochemical approach to the isolation and characterization of B lymphocyte antigen-receptors using *net* anti-Ig-sensitive HCY binding as a means of assay. The most promising method would be to label the cells with radioactive antigen *before* cell disruption, as binding of antigen to intracellular components, particularly intracellular Ig, will then be minimized. Furthermore, cell surface components could be radioiodinated with ¹²⁵I (using lactoperoxidase, or possibly chloramine-T) before or after incubation of cells with ¹³¹I-labeled HCY. There is a reasonable chance that HCY may preferentially inhibit the radioiodination, especially by lactoperoxidase, of the surface component(s) which are isolated as complexes with HCY.

There are a number of methods by which the (HCY/receptor) complexes might be purified. Firstly their size could be utilized, as was done for (Ig/anti-Ig) complexes in Chapter 6. However, this type of study requires that all the complexes be similar in size, *i.e.* aggregation, either by cross-linkage of receptors or polymerization of receptor-bound antigen, must be prevented. The effect of iodination/oxidation on the ability of HCY to polymerize at pH 7.2 was studied for this reason, but this treatment did not prevent polymerization. Polymerization of HCY might be circumvented however if labeled, pH 9-depolymerized HCY is added at low concentration to cells at pH 7.2. The polymerization is slow (attainment of equilibrium requires > 10 h at 20°C; Moore, 1967) and is unlikely to occur significantly during cell labeling (60 min., 0°C) or after all unbound HCY (> 95 % of total added to cells) is washed away.

Secondly, complexes could be precipitated with specific antisera. Thus the components precipitated by anti-HCY serum or by anti-Ig sera could be analyzed.

Thirdly, use could be made of the capping phenomenon, as has been done for surface-Ig by Melchers and Andersson (1973). Sufficient HCY could be added to cells to saturate all HCY-receptors and prevent their cross-linking. The cells, still in HCY solution, could then be incubated at 37°C in the presence of anti-Ig at a concentration which results in optimal capping of surface-Ig. The HCY should prevent HCV-saturated receptors (if they are Ig) from capping. Thus, most surface-Ig would be removed as an insoluble aggregate upon cell solution.

These techniques, either singly or in combination, may for the first time allow direct isolation of an antigen receptor, rather than an Ig molecule or other surface component which is *presumed* to possess receptor activity.

7.4 Summary

The ability of anti-IgM serum to inhibit the *net* binding of a number of different ^{125}I -labeled antigens to mouse and sheep lymphoid cell populations was tested. Haemocyanin was the only antigen whose binding was reduced by more than 35 % with respect to binding to cells treated with normal rabbit serum. The maximum reduction in net binding of haemocyanin to mouse spleen cells was 84 %.

The net amount of haemocyanin bound to mouse spleen cells pretreated with either normal rabbit or anti-mouse-IgM serum was measured after the cells were incubated with the ^{125}I -labeled antigen for different periods of time (0-135 min.) or at a number of antigen concentrations (0-1 $\mu\text{g/ml}$). Neither of these parameters significantly affected the proportion of cell-associated haemocyanin whose binding was anti-IgM-sensitive.

The large difference in net binding of haemocyanin to anti-IgM-pretreated compared to normal rabbit serum-pretreated mouse spleen cells is suggested to provide a means of identifying, by its direct isolation as a complex with haemocyanin, the immunologically-important receptor on haemocyanin-specific antigen-binding lymphocytes.

Chapter 8

General discussion and conclusions

At the time this study was commenced, it was known that (a) antigens which possessed a restricted number of determinants were bound to the surface of only a proportion of lymphocytes from normal animals (Ada, 1970) and (b) that the receptors which bound these antigens could be "blocked" by treating the cells with anti-Ig sera before exposing them to antigen (e.g. Warner, Byrt and Ada, 1970).

The aim of this thesis was to establish, if possible by their isolation and direct characterization, the identity of the (B) lymphocyte receptors for antigen. However, biochemical isolation of these receptors posed problems not normally encountered in the isolation of biological macromolecules possessing affinities for given substrates. The first of these problems was the heterogeneity of the receptors - only a small proportion of the receptors present on an unfractionated population of lymphocytes bind a given antigen, whereas *all* the molecules of an enzyme, for example, can be assayed by their individual affinities for the *same* substrate. Secondly, the receptors bind noncovalently to antigens, and the complexes so formed could be isolated only in conditions which would not cause them to dissociate.

There seemed a number of ways in which the receptors might be isolated and identified. Firstly, if the plasma membranes of intact cells could be preferentially and covalently labeled with (for instance) a protein reagent, the cells could be disrupted, the plasma membranes isolated and the membrane components separated. Thus those components which (in the intact cell) were exposed to the extracellular medium could be identified. Secondly, labeled antigens could be bound to the cells and the plasma membranes then isolated and fractionated under non-dissociating conditions. In this way, the (receptor/antigen) complexes might be characterized. Thirdly, anti-Ig reagents could be employed to inhibit the labeling of the receptors by antigens or by other reagents.

To implement these ideas, the following studies were undertaken:

1. Intact red blood cells were radioiodinated and then disrupted to determine whether their plasma membranes could be selectively labeled (Chapter 3).

2. Sheep lymphocytes were fractionated with the aim of establishing a procedure for the isolation of their plasma membranes (Chapter 4).
3. The properties of the immunoglobulins present on the surface of sheep lymphocytes were investigated (Chapters 5 and 6), as these molecules were thought likely to be the receptors for antigen (see Chapter 1).
4. The ability of anti-Ig sera to inhibit net binding of antigens to lymphocyte populations was determined with the aim of isolating the receptors in the form of complexes with labeled antigen (Chapter 7).

The remainder of this discussion is a summary of the major findings and conclusions of these aspects:

1. The use of chloramine-T to label intact red blood cells with radioactive iodide resulted in very selective iodination of their plasma membranes. This was shown for SRBC, whose membranes were labeled to a specific (protein) radioactivity some 500-600 fold higher than that of haemoglobin. A similar result was obtained for CRBC, a more complicated system than SRBC because these cells are nucleated.

During the course of this work, the use of lactoperoxidase to selectively radioiodinate the plasma membranes of intact human red blood cells was reported by Phillips and Morrison (1970). Others have since reported using this technique to label plasma membranes of human RBC (Hubbard and Cohn, 1972) or lymphoid cells (Marchalonis, Cone and Santer, 1971; Baur, Vitetta, Sherr, Schenkein and Uhr, 1971). Because lactoperoxidase is a large protein (MW:77,000 daltons), it can catalyze the iodination of only those tyrosine residues which are readily accessible on the external surface of the cell. However, chloramine-T-mediated iodination, which should not be subject to such strict steric limitations, selectively labeled SRBC membranes to nearly the same degree that lactoperoxidase labeled the membranes of HRBC. The two methods, used in conjunction, may therefore be useful for investigating the accessibility of proteins on cell surfaces.

2. Fractionation of sheep lymphocytes labeled before disruption with ^{125}I -labeled antibodies specific for surface antigens revealed that in the presence of Mg^{++} , labeled plasma membrane fragments were heterogeneous both in size and density, whereas at low ionic strength and in the presence of EDTA the labeled material was more homogeneous. Although this effect of EDTA was probably due to the removal of cations from the membrane fragments, the changes resulting from such depletion are not understood. It was clear from this work that the isolation of plasma membranes from lymphocytes is a more complicated procedure than is that from RBC, and that great care must be taken in regard to the use of valid markers and the possible selective loss of components.

It was also found that treatment of antibody-labeled lymphocytes with NP40 disrupted the labeled membranes to a soluble form, without dissociating the antibodies from cellular antigens. Because the complexes obtained in NP40-extracts of ALG-labeled cells differed in size from those obtained in similar extracts of anti-L-chain-labeled cells, it seemed that the size of the complexes depended on the components to which the antibodies were bound. Subsequent use was made of this phenomenon in investigating the size and fate of the complexes formed at the lymphocyte surface by the binding of anti-Ig antibodies to surface-Ig.

3. Sheep efferent popliteal lymph cell populations were shown to contain at least two distinct types of lymphocyte. One kind of cell possesses large quantities of μ and light (Ig) chain determinants on its surface. These determinants are present as 7S and 19S IgM molecules as judged by the sedimentation coefficients of their complexes with anti-Ig antibodies. The other type of lymphocyte does not contain surface-Ig in detectable quantities as determined in this study.

Except for the demonstration of 19S IgM, these findings are consistent with those of others working with mice and chickens (see Chapter 1) and by analogy with these animals, sheep Ig^+ lymphocytes could be expected to be nonthymus-derived (B) cells and Ig^- lymphocytes, thymus-derived (T) cells. The presence of 19S IgM on the surface of sheep lymphocytes might perhaps be due to the presence of "memory" B cells in the cell

population, for Melchers and Andersson (1973) have reported that unstimulated mouse B lymphocytes contain 7S (but not 19S) IgM on their surfaces, whereas after prolonged mitogenic stimulation 19S IgM begins to appear on the cell surface. This might be investigated by characterizing the relative proportions of 7S and 19S IgM on the surfaces of lymphocytes from sheep of different ages or after immunization with antigen.

4. Although anti-Ig sera are reported from autoradiographic studies to significantly inhibit the binding of labeled antigens (such as *Salmonella* flagellin) to specific antigen-binding lymphocytes (e.g. Warner, Byrt and Ada, 1970), preliminary experiments reported in this thesis have shown that such sera are less able to inhibit the net uptake of these antigens to lymphoid cell populations. The inhibition resulting from such treatment depended on the particular antigen used and the binding was in some cases *enhanced* rather than reduced. However, the net binding of haemocyanin, in contrast to that of the other antigens whose binding was tested, was consistently inhibited by 70-80 % if the cells were exposed beforehand to anti-Ig serum. Haemocyanin therefore bound mainly to anti-Ig-sensitive sites on mouse lymphoid cell populations. This finding shows promise in that it might, for the first time, allow (by the isolation of an (antigen/receptor) complex) the direct identification of lymphocyte receptors which bind a single antigen. Furthermore, by using radioactive antigen-mediated "suicide" of mouse lymphoid cells, these receptors might be shown to be functional in cellular stimulation by antigen. Work is being continued on these aspects.

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